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Patent Docket P1101P1

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| In re Application of<br>Avi J. Ashkenazi<br>Serial No.: 09/020,746<br>Filed: February 9, 1998<br>For: Apo-2 Receptor Antibody | Group Art Unit: 1647<br>Examiner: C. Kaufman |

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**RULE 131 DECLARATION**

I, Avi J. Ashkenazi, hereby declare as follows:

1. I am the named inventor of the claimed subject matter of the above-identified patent application.

2. The above-identified patent application claims priority to application serial no. 08/857,216 filed with the Patent Office on May 15, 1997, and I am the named inventor in that priority application. A copy of my priority application serial no. 08/857,216 is attached as Exhibit A.

3. All work described in the above-identified application and the priority application (Exhibit A) was performed by me or on my behalf in the United States of America.

4. I have read and reviewed US Patent 6,072,047 issued to Rauch et al. on June 6, 2000 (hereinafter the "'047 patent") (a copy of which is attached as Exhibit B). I understand that the '047 patent is based on application serial no. 08/883,036 filed with the Patent Office on June 26, 1997 and claims priority to four different priority patent applications filed in the Patent Office in the time period between February 13, 1997 and June 26, 1997.

5. I have also read and reviewed the following four priority patent applications that are referred to in the '047 patent: No. 08/869,852 filed June 4, 1997 (attached as Exhibit C); No. 08/829,536 filed March 28, 1997 (attached as Exhibit D); No.

08/815,255 filed March 12, 1997 (attached as Exhibit E); and No. 08/799,861 filed February 13, 1997 (attached as Exhibit F).

6. The priority application (Exhibit A) filed on my behalf on May 15, 1997 demonstrates both my conception of the claimed invention of the present application and a constructive reduction to practice of the invention prior to at least the June 4, 1997 priority filing date of the '047 patent.

7. Experiments performed by me or on my behalf relating to the identification and structural characterization of the Apo-2 receptor are described, for example, in Example 1 of Exhibit A, pages 58-62. In *in vitro* binding assays, I found that the Apo-2 receptor extracellular domain binds the ligand known as Apo-2 ligand (Exhibit A, e.g., pages 63, lines 9-35 - page 64, lines 1-6). In further *in vitro* assays, I also found that the Apo-2 receptor was capable of inducing cell death in transfected mammalian cells (Exhibit A, page 64, lines 9-35 - page 65, lines 1 -13).

8. In Exhibit A, agonist antibodies to the Apo-2 receptor are described. (See, e.g., Page 10, lines 3-5; Page 15, lines 7-10; Page 56, lines 21-23). More particularly, Exhibit A discloses that an agonistic Apo-2 antibody may be employed to activate or stimulate apoptosis in mammalian cancer cells (Page 56, lines 21-23). Methods for making Apo-2 antibodies are described on pages 48-56 of Exhibit A. Apoptotic activity in mammalian cells is described on, e.g., page 17, lines 1-12, of Exhibit A.

9. Exhibit A therefore demonstrates that agonist antibodies which bind Apo-2 receptor and stimulate apoptosis were conceived and constructively reduced to practice by the May 15, 1997 filing date of my patent application. This May 15, 1997 date is prior to the date of the '047 patent priority application filed June 4, 1997.

10. Upon completing my review of the '047 patent (Exhibit B), I conclude that the only reference in the '047 patent concerning antibodies that can be raised that have agonist function for TRAIL-R is contained in a single paragraph in Column 21, lines 35-46 (and reproduced as follows):

Antibodies raised against TRAIL-R may be screened for agonistic (i.e., ligand-mimicking) properties. Such antibodies, upon binding to cell surface TRAIL-R, induce biological effects (e.g., transduction of biological signals) similar to the biological effects induced when TRAIL binds to cell surface TRAIL-R. Agonistic antibodies may be used to induce apoptosis of certain cancer cells or virally infected cells, as has been reported for TRAIL. The ability of TRAIL to kill cancer cells (including but not limited to leukemia, lymphoma, and melanoma cells) and virally infected cells is described in Wiley et al. (Immunity 3:673-682, 1995); and in PCT application WO 97/01633.

This paragraph is not found in any of the other '047 patent priority applications that have a filing date prior to May 15, 1997 (Exhibits D-F).

11. In comparing the disclosures of the '047 patent and its four priority applications, I found that the text cited in above paragraph 10 was added for the first time in the '047 patent's priority application filed June 4, 1997 (Exhibit C, Page 27, lines 3-10). The other priority applications do not include this paragraph, which is clear from a comparison of the surrounding text of the March 28, 1997 priority application (Exhibit D) at page 20, lines 1-21.

12. The '047 patent's remaining three priority applications (Exhibits D, E, and F) filed March 28, 1997, March 12, 1997 and February 13, 1997, respectively, do not include this paragraph (recited in paragraph 10 above) and do not include any description of an agonist antibody or of use(s) of an agonist antibody.

13. Neither the '047 patent (Exhibit B) nor any of its four priority applications (Exhibits C-F) identify or describe any examples of hybridomas or monoclonal antibodies that were actually produced. There is likewise no description in the '047 patent (Exhibit B) or any of the four priority applications (Exhibits C-F) of any example of a monoclonal antibody that was actually produced which binds to Apo-2/TRAIL-R, or which has apoptotic, or ligand-mimicking, activity.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and

belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

10/15/02
Date

Avi J Ashkenazi
Avi J. Ashkenazi, Ph.D.

# EXHIBIT A

PATENT DOCKET NO. P1101
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# Apo-2 Receptor

## FIELD OF THE INVENTION

The present invention relates generally to the identification, isolation, and recombinant production of novel polypeptides, designated herein as "Apo-2".

## BACKGROUND OF THE INVENTION

### Apoptosis or "Programmed Cell Death"

Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, *e.g.*, Barr et al., Bio/Technology, 12:487-493 (1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels of apoptotic cell death have been associated with a variety of pathological conditions, including cancer, lupus, and herpes virus infection [Thompson, Science, 267:1456-1462 (1995)]. Increased levels of apoptotic cell death may be associated with a variety of other pathological conditions, including AIDS, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic anemia, myocardial infarction, stroke, reperfusion injury, and toxin-induced liver disease [see, Thompson, supra].

Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra; Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as *myc*, *rel*, and *E1A*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been observed to have apoptosis-inducing activity [Thompson, supra].

TNF Family of Cytokines

Various molecules, such as tumor necrosis factor-$\alpha$ ("TNF-$\alpha$"), tumor necrosis factor-$\beta$ ("TNF-$\beta$" or "lymphotoxin"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also referred to as TRAIL) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995); Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996)]. Among these molecules, TNF-$\alpha$, TNF-$\beta$, CD30 ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2 ligand (TRAIL) have been reported to be involved in apoptotic cell death. Both TNF-$\alpha$ and TNF-$\beta$ have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987)]. Zheng et al. have reported that TNF-$\alpha$ is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported that CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory

Symposium on Programmed Cell Death, Abstr. No. 10, (1995)].

Mutations in the mouse Fas/Apo-1 receptor or ligand genes (called *lpr* and *gld*, respectively) have been associated with some autoimmune disorders, indicating that Apo-1 ligand may play a role in regulating the clonal deletion of self-reactive lymphocytes in the periphery [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata et al., Science, 267:1449-1456 (1995)]. Apo-1 ligand is also reported to induce post-stimulation apoptosis in CD4-positive T lymphocytes and in B lymphocytes, and may be involved in the elimination of activated lymphocytes when their function is no longer needed [Krammer et al., supra; Nagata et al., supra]. Agonist mouse monoclonal antibodies specifically binding to the Apo-1 receptor have been reported to exhibit cell killing activity that is comparable to or similar to that of TNF-$\alpha$ [Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

## TNF Family of Receptors

Induction of various cellular responses mediated by such TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991] and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized [Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive polymorphisms have been associated with both TNF receptor genes [see, e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions. The extracellular portions of both receptors are found naturally also as soluble TNF-binding proteins [Nophar, Y. et al., EMBO J., 9:3269 (1990); and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A.,

87:8331 (1990)]. More recently, the cloning of recombinant soluble TNF receptors was reported by Hale et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

The extracellular portion of type 1 and type 2 TNFRs (TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern of four cysteine-rich domains (CRDs) designated 1 through 4, starting from the $NH_2$-terminus. Each CRD is about 40 amino acids long and contains 4 to 6 cysteine residues at positions which are well conserved [Schall et al., supra; Loetscher et al., supra; Smith et al., supra; Nophar et al., supra; Kohno et al., supra]. In TNFR1, the approximate boundaries of the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2- amino acids from about 54 to about 97; CRD3- amino acids from about 98 to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2, CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about 55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4- amino acids from about 141 to about 179 [Banner et al., Cell, 73:431-435 (1993)]. The potential role of the CRDs in ligand binding is also described by Banner et al., supra.

A similar repetitive pattern of CRDs exists in several other cell-surface proteins, including the p75 nerve growth factor receptor (NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature, 325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J., 8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 9:1063 (1990)] and the Fas antigen [Yonehara et al., supra and Itoh et al., supra]. CRDs are also found in the soluble TNFR (sTNFR)-like T2 proteins of the Shope and myxoma poxviruses [Upton et al., Virology, 160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335 (1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of these sequences indicates that the positions of the cysteine residues are well conserved. These receptors are sometimes collectively referred to as members of the TNF/NGF receptor superfamily. Recent studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid insertion in

this domain [Yan, H. and Chao, M.V., J. Biol. Chem., 266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H. and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of about 60 amino acids, between its CRD4 and transmembrane region, which is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol., 41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973 (1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region is found in TNFR2 but not in TNFR1.

Itoh et al. disclose that the Apo-1 receptor can signal an apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNFR1 when cells are treated with either TNF-$\alpha$ or anti-Apo-1 mouse monoclonal antibody [Krammer et al., supra; Nagata et al., supra]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNFR1 receptors may mediate cell killing through common signaling pathways [Id.].

The TNF family ligands identified to date, with the exception of lymphotoxin-$\alpha$, are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, the receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF-$\alpha$, Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines.

Recently, other members of the TNFR family have been identified. In Marsters et al., Curr. Biol., 6:750 (1996), investigators describe a full length native sequence human polypeptide, called Apo-3, which exhibits similarity to the TNFR

family in its extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that it contains a cytoplasmic death domain sequence [see also Marsters et al., Curr. Biol., 6:1669 (1996)]. Apo-3 has also been referred to by other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., Science, 274:990 (1996); Kitson et al., Nature, 384:372 (1996); Bodmer et al., Immunity, 6:79 (1997)].

Pan et al. have disclosed another TNF receptor family member referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4 was reported to contain a cytoplasmic death domain capable of engaging the cell suicide apparatus. Pan et al. disclose that DR4 is believed to be a receptor for the ligand known as Apo-2 ligand or TRAIL.

The Apoptosis-Inducing Signaling Complex

As presently understood, the cell death program contains at least three important elements - activators, inhibitors, and effectors; in *C. elegans*, these elements are encoded respectively by three genes, *Ced-4*, *Ced-9* and *Ced-3* [Steller, Science, 267:1445 (1995); Chinnaiyan et al., Science, 275:1122-1126 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, Current Biology, 6:555-562 (1996); Fraser and Evan, Cell; 85:781-784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF-$\kappa$B [Tartaglia et al., Cell, 74:845-853 (1993); Hsu et al., Cell, 84:299-308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., supra; Nagata, Cell, 88:355 (1997)]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the Drosophila protein, Reaper, and the mammalian proteins referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, Cell, 81:479-482 (1995)]. Using the yeast-two hybrid system, Raven et al. report the identification of protein, wsl-1, which binds to the TNFR1 death domain [Raven et al., Programmed Cell Death Meeting, September 20-24, 1995, Abstract at page 127; Raven et al., European Cytokine Network, 7:Abstr. 82 at page 210 (April-June 1996)]. The

wsl-1 protein is described as being homologous to TNFR1 (48% identity) and having a restricted tissue distribution. According to Raven et al., the tissue distribution of wsl-1 is significantly different from the TNFR1 binding protein, TRADD.

Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signalling complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., Cell, 81:505-512 (1995); Boldin et al., J. Biol. Chem., 270:387-391 (1995); Hsu et al., supra; Chinnaiyan et al., J. Biol. Chem., 271:4961-4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the *Ced-3*-related protease, MACH$\alpha$/FLICE (caspase 8), into the death signalling complex [Boldin et al., Cell, 85:803-815 (1996); Muzio et al., Cell, 85:817-827 (1996)]. MACH$\alpha$/FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1$\beta$ converting enzyme (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death programme [Fraser and Evan, supra].

It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, *ced-3*, and to the mammalian IL-1-converting enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, *crmA* [Ray et al., Cell, 69:597-604 (1992); Tewari et al., Cell, 81:801-809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., Nature, 375:78-81 (1995); Tewari et al., J. Biol. Chem., 270:3255-3260 (1995)].

As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF-$\kappa$B [Tewari et al., Curr. Op. Genet. Develop., 6:39-44 (1996)]. NF-$\kappa$B is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., Genes Develop., 9:2723-2735

(1996); Baldwin, Ann. Rev. Immunol., 14:649-681 (1996)]. In its latent form, NF-κB is complexed with members of the IκB inhibitor family; upon inactivation of the IκB in response to certain stimuli, released NF-κB translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription.

For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, supra.

## SUMMARY OF THE INVENTION

Applicants have identified cDNA clones that encode novel polypeptides, designated in the present application as "Apo-2." It is believed that Apo-2 is a member of the TNFR family; full-length native sequence human Apo-2 polypeptide exhibits some similarities to some known TNFRs, including a cytoplasmic death domain region. Full-length native sequence human Apo-2 also exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats. Apo-2 polypeptide has been found to be capable of triggering caspase-dependent apoptosis and activating NF-κB. Applicants surprisingly found that the soluble extracellular domain of Apo-2 binds Apo-2 ligand (Apo-2L) and can inhibit Apo-2 ligand function. It is presently believed that Apo-2 ligand can signal via at least two different receptors, DR4 and the newly described Apo-2 herein.

In one embodiment, the invention provides isolated Apo-2 polypeptide. In particular, the invention provides isolated native sequence Apo-2 polypeptide, which in one embodiment, includes an amino acid sequence comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). In other embodiments, the isolated Apo-2 polypeptide comprises at least about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1).

In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally, the isolated extracellular domain sequence comprises amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides an isolated

death domain sequence of Apo-2. Optionally, the isolated death domain sequence comprises amino acid residues 324 to 391 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides chimeric molecules comprising Apo-2 polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an Apo-2 fused to an immunoglobulin sequence. Another example comprises an extracellular domain sequence of Apo-2 fused to a heterologous polypeptide or amino acid sequence, such as an immunoglobulin sequence.

In another embodiment, the invention provides an isolated nucleic acid molecule encoding Apo-2 polypeptide. In one aspect, the nucleic acid molecule is RNA or DNA that encodes an Apo-2 polypeptide or a particular domain of Apo-2, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. In one embodiment, the nucleic acid sequence is selected from:

(a) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 411 (i.e., nucleotides 140-142 through 1370-1372), inclusive;

(b) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 182 (i.e., nucleotides 140-142 through 683-685), inclusive;

(c) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 54 to residue 182 (i.e., nucleotides 299-301 through 683-685), inclusive;

(d) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 324 to residue 391 (i.e., nucleotides 1109-1111 through 1310-1312), inclusive; or

(e) a sequence corresponding to the sequence of (a), (b), (c) or (d) within the scope of degeneracy of the genetic code.

In a further embodiment, the invention provides a vector comprising the nucleic acid molecule encoding the Apo-2 polypeptide or particular domain of Apo-2. A host cell comprising the vector

or the nucleic acid molecule is also provided. A method of producing Apo-2 is further provided.

In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an agonistic, antagonistic or neutralizing antibody.

In another embodiment, the invention provides non-human, transgenic or knock-out animals.

A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 or Apo-2 antibodies.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of a native sequence human Apo-2 cDNA and its derived amino acid sequence.

Figure 2A shows the derived amino acid sequence of a native sequence human Apo-2 - the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined.

Figure 2B shows an alignment and comparison of the death domain sequences of native sequence human Apo-2, DR4, Apo-3/DR3, TNFR1, and Fas/Apo-1 (CD95). Asterisks indicate residues that are essential for death signaling by TNFR1 [Tartaglia et al., supra].

Figure 3 shows the interaction of the Apo-2 ECD with Apo-2L. Supernatants from mock-transfected 293 cells or from 293 cells transfected with Flag epitope-tagged Apo-2 ECD were incubated with poly-His-tagged Apo-2L and subjected to immunoprecipitation with anti-Flag conjugated or Nickel conjugated agarose beads. The precipitated proteins were resolved by electrophoresis on polyacrylamide gels, and detected by immunoblot with anti-Apo-2L or anti-Flag antibody.

Figure 4 shows the induction of apoptosis by Apo-2 and inhibition of Apo-2L activity by soluble Apo-2 ECD. Human 293 cells (A, B) or HeLa cells (C) were transfected by pRK5 vector or by pRK5-based plasmids encoding Apo-2 and/or CrmA. Apoptosis was assessed by morphology (A), DNA fragmentation (B), or by FACS (C-

E). Soluble Apo-2L was pre-incubated with buffer or affinity-purified Apo-2 ECD together with anti-Flag antibody or Apo-2 ECD immunoadhesin or DR4 or TNFR1 immunoadhesins and added to HeLa cells. The cells were later analyzed for apoptosis (D). Dose-response analysis using Apo-2L with Apo-2 ECD immunoadhesin was also determined (E).

Figure 5 shows activation of NF-κB by Apo-2, DR4, and Apo-2L. (A) HeLa cells were transfected with expression plasmids encoding the indicated proteins. Nuclear extracts were prepared and analyzed by an electrophoretic mobility shift assay. (B) HeLa cells or MCF7 cells were treated with buffer, Apo-2L or TNF-alpha and assayed for NF-κB activity. (C) HeLa cells were preincubated with buffer, ALLN or cyclohexamide before addition of Apo-2L. Apoptosis was later analyzed by FACS.

Figure 6 shows expression of Apo-2 mRNA in human tissues as analyzed by Northern hybridization of human tissue poly A RNA blots.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

### I. Definitions

The terms "Apo-2 polypeptide" and "Apo-2" when used herein encompass native sequence Apo-2 and Apo-2 variants (which are further defined herein). These terms encompass Apo-2 from a variety of mammals, including humans. The Apo-2 may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

A "native sequence Apo-2" comprises a polypeptide having the same amino acid sequence as an Apo-2 derived from nature. Thus, a native sequence Apo-2 can have the amino acid sequence of naturally-occurring Apo-2 from any mammal. Such native sequence Apo-2 can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-2" specifically encompasses naturally-occurring truncated or secreted forms of the Apo-2 (*e.g.*, an extracellular domain sequence), naturally-occurring variant forms (*e.g.*, alternatively spliced forms) and naturally-

occurring allelic variants of the Apo-2. A naturally-occurring variant form of the Apo-2 includes an Apo-2 having an amino acid substitution at residue 410 in the amino acid sequence shown in Figure 1 (SEQ ID NO:1). In one embodiment of such naturally-ocurring variant form, the leucine residue at position 410 is substituted by a methionine residue. In Fig. 1 (SEQ ID NO:1), the amino acid residue at position 410 is identified as "Xaa" to indicate that the amino acid may, optionally, be either leucine or methionine. In Fig. 1 (SEQ ID NO:2), the nucleotide at position 1367 is identified as "W" to indicate that the nucleotide may be either adenine (A) or thymine (T) or uracil (U). In one embodiment of the invention, the native sequence Apo-2 is a mature or full-length native sequence Apo-2 comprising amino acids 1 to 411 of Fig. 1 (SEQ ID NO:1).

The "Apo-2 extracellular domain" or "Apo-2 ECD" refers to a form of Apo-2 which is essentially free of the transmembrane and cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally, Apo-2 ECD will comprise amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1) or amino acid residues 1 to 182 of Fig. 1 (SEQ ID NO:1).

"Apo-2 variant" means a biologically active Apo-2 as defined below having at least about 80% amino acid sequence identity with the Apo-2 having the deduced amino acid sequence shown in Fig. 1 (SEQ ID NO:1) for a full-length native sequence human Apo-2. Such Apo-2 variants include, for instance, Apo-2 polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the sequence of Fig. 1 (SEQ ID NO:1). Ordinarily, an Apo-2 variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95% amino acid sequence identity with the amino acid sequence of Fig. 1 (SEQ ID NO:1).

"Percent (%) amino acid sequence identity" with respect

to the Apo-2 sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the Apo-2 sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN™ or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising Apo-2, or a domain sequence thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the Apo-2. The tag polypeptide preferably also is fairly unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning

cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the Apo-2 natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated" Apo-2 nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the Apo-2 nucleic acid. An isolated Apo-2 nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2 nucleic acid molecules therefore are distinguished from the Apo-2 nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2 nucleic acid molecule includes Apo-2 nucleic acid molecules contained in cells that ordinarily express Apo-2 where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that

the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers single anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) and anti-Apo-2 antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 antibody with a constant domain (*e.g.* "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (*e.g.*, Fab, $F(ab')_2$, and Fv), so long as they exhibit the desired biological activity. See, *e.g.* U.S. Pat. No. 4,816,567 and Mage et al., in <u>Monoclonal Antibody Production Techniques and Applications</u>, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous

population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

"Humanized" forms of non-human (*e.g.* murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

"Biologically active" and "desired biological activity" for the purposes herein mean having the ability to modulate

apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell *in vivo* or *ex vivo*.

The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.

The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

## II. <u>Compositions and Methods of the Invention</u>

The present invention provides newly identified and isolated Apo-2 polypeptides. In particular, Applicants have identified and isolated various human Apo-2 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides are described in further detail in the Examples below. Based upon the properties and characteristics of the Apo-2 polypeptides disclosed herein, it is Applicants' present belief that Apo-2 is a member of the TNFR family.

A description follows as to how Apo-2, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared.

A. Preparation of Apo-2

The description below relates primarily to production of Apo-2 by culturing cells transformed or transfected with a vector containing Apo-2 nucleic acid. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare Apo-2.

1. Isolation of DNA Encoding Apo-2

The DNA encoding Apo-2 may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 mRNA and to express it at a detectable level. Accordingly, human Apo-2 DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage libraries of human pancreas and kidney cDNA described in Example 1. The Apo-2-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the Apo-2 or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer:A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

A preferred method of screening employs selected oligonucleotide sequences to screen cDNA libraries from various human tissues. Example 1 below describes techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like $^{32}$P-labeled ATP, biotinylation or enzyme labeling. Hybridization conditions,

inlcuding moderate stringency and high stringency, are provided in Sambrook et al., supra.

Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Apo-2 variants can be prepared by introducing appropriate nucleotide changes into the Apo-2 DNA, or by synthesis of the desired Apo-2 polypeptide. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the Apo-2, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics.

Variations in the native full-length sequence Apo-2 or in various domains of the Apo-2 described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the Apo-2 that results in a change in the amino acid sequence of the Apo-2 as compared with the native sequence Apo-2. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the Apo-2 molecule. The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the Apo-2 variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence which are involved in the interaction with a particular ligand or receptor. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is the preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

Once selected Apo-2 variants are produced, they can be contacted with, for instance, Apo-2L, and the interaction, if any, can be determined. The interaction between the Apo-2 variant and Apo-2L can be measured by an *in vitro* assay, such as described in the Examples below. While any number of analytical measurments can be used to compare activities and properties between a native sequence Apo-2 and an Apo-2 variant, a convenient one for binding is the dissociation constant $K_d$ of the complex formed between the Apo-2 variant and Apo-2L as compared to the $K_d$ for the native sequence Apo-2. Generally, a $\geq$ 3-fold increase or decrease in $K_d$ per substituted residue indicates that the substituted residue(s) is active in the interaction of the native sequence Apo-2 with the Apo-2L.

Optionally, representative sites in the Apo-2 sequence suitable for mutagenesis would include sites within the extracellular domain, and particularly, within one or both of the cysteine-rich domains. Such variations can be accomplished using the methods described above.

2. Insertion of Nucleic Acid into A Replicable Vector

The nucleic acid (*e.g.*, cDNA or genomic DNA) encoding Apo-2 may be inserted into a replicable vector for further cloning

(amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

(i) Signal Sequence Component

The Apo-2 may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, *e.g.*, the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders, the latter described in U.S. Pat. No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native Apo-2 presequence that normally directs insertion of Apo-2 in the cell membrane of human cells *in vivo* is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.

The DNA for such precursor region is preferably ligated in reading frame to DNA encoding Apo-2.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2$\mu$ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used because it contains the early promoter).

Most expression vectors are "shuttle" vectors, *i.e.*, they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of Apo-2 DNA. However, the recovery of genomic DNA encoding Apo-2 is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the Apo-2 DNA.

(iii) Selection Gene Component

Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host

cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al., J. Molec. Appl. Genet., 1:327 (1982)], mycophenolic acid (Mulligan et al., Science, 209:1422 (1980)] or hygromycin [Sugden et al., Mol. Cell. Biol., 5:410-413 (1985)]. The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the Apo-2 nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes Apo-2. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of Apo-2 are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, adenosine deaminase, and

ornithine decarboxylase.

Cells transformed with the DHFR selection gene may first be identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding Apo-2. This amplification technique can be used with any otherwise suitable host, *e.g.*, ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding Apo-2, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, *e.g.*, kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)]. The presence of the *trp*1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu*2 gene.

In addition, vectors derived from the 1.6 $\mu$m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts [Bianchi et al., Curr. Genet., 12:185 (1987)]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den Berg, Bio/Technology, 8:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed [Fleer et al., Bio/Technology, 9:968-975 (1991)].

(iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the Apo-2 nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, *e.g.*, the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Apo-2 promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 DNA.

Promoters suitable for use with prokaryotic hosts include the $\beta$-lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-

25 (1983)]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding Apo-2 [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Apo-2.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Apo-2 transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the Apo-2 sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication [Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978 [See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature, 297:598-601 (1982) on expression of human $\beta$-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human interferon $\beta$1 gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter].

(v) Enhancer Element Component

Transcription of a DNA encoding the Apo-2 of this

invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5′ [Laimins et al., Proc. Natl. Acad. Sci. USA, 78:993 (1981]) and 3′ [Lusky et al., Mol. Cell Bio., 3:1108 (1983]) to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself [Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5′ or 3′ to the Apo-2 coding sequence, but is preferably located at a site 5′ from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5′ and, occasionally 3′, untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding Apo-2.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the

plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxam et al., Methods in Enzymology, 65:499 (1980).

(viii) Transient Expression Vectors

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2 may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., *supra*]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying Apo-2 variants.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2 in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

3. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or

Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia, e.g., E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., Salmonella typhimurium, Serratia, e.g., Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g., B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

Suitable host cells for the expression of glycosylated Apo-2 are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified [See, *e.g.*, Luckow et al., <u>Bio/Technology</u>, <u>6</u>:47-55 (1988); Miller et al., in <u>Genetic Engineering</u>, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., <u>Nature</u>, <u>315</u>:592-594 (1985)]. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the Apo-2

can be transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the Apo-2-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences [Depicker et al., J. Mol. Appl. Gen., 1:561 (1982)]. In addition, DNA segments isolated from the upstream region of the T-DNA *780* gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue [EP 321,196 published 21 June 1989].

Propagation of vertebrate cells in culture (tissue culture) is also well known in the art [See, *e.g.*, Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; and FS4 cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2 production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in

fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, *e.g.*, polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

4. Culturing the Host Cells

Prokaryotic cells used to produce Apo-2 may be cultured in suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce Apo-2 may be

cultured in a variety of media. Examples of commercially available media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly $^{32}P$. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively,

antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, or luminescent labels.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence Apo-2 polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to Apo-2 DNA and encoding a specific antibody epitope.

6. Purification of Apo-2 Polypeptide

Forms of Apo-2 may be recovered from culture medium or from host cell lysates. If the Apo-2 is membrane-bound, it can be released from the membrane using a suitable detergent solution (*e.g.* Triton-X 100) or its extracellular domain may be released by enzymatic cleavage.

When Apo-2 is produced in a recombinant cell other than one of human origin, the Apo-2 is free of proteins or polypeptides of human origin. However, it may be desired to purify Apo-2 from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. Apo-2 thereafter is purified from

contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Apo-2 variants in which residues have been deleted, inserted, or substituted can be recovered in the same fashion as native sequence Apo-2, taking account of changes in properties occasioned by the variation. For example, preparation of an Apo-2 fusion with another protein or polypeptide, *e.g.*, a bacterial or viral antigen, immunoglobulin sequence, or receptor sequence, may facilitate purification; an immunoaffinity column containing antibody to the sequence can be used to adsorb the fusion polypeptide. Other types of affinity matrices also can be used.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native sequence Apo-2 may require modification to account for changes in the character of Apo-2 or its variants upon expression in recombinant cell culture.

7. <u>Covalent Modifications of Apo-2 Polypeptides</u>

Covalent modifications of Apo-2 are included within the scope of this invention. One type of covalent modification of the Apo-2 is introduced into the molecule by reacting targeted amino acid residues of the Apo-2 with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of the Apo-2.

Derivatization with bifunctional agents is useful for crosslinking Apo-2 to a water-insoluble support matrix or surface for use in the method for purifying anti-Apo-2 antibodies, and

vice-versa. Derivatization with one or more bifunctional agents will also be useful for crosslinking Apo-2 molecules to generate Apo-2 dimers. Such dimers may increase binding avidity and extend half-life of the molecule *in vivo*. Commonly used crosslinking agents include, *e.g.*, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl-propionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)-dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the $\alpha$-amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The modified forms of the residues fall within the scope of the present invention.

Another type of covalent modification of the Apo-2 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence Apo-2, and/or adding one or more glycosylation sites that are not present in the native sequence Apo-2.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the Apo-2 polypeptide may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native sequence Apo-2 (for O-linked glycosylation sites). The Apo-2 amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apo-2 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.

Another means of increasing the number of carbohydrate moieties on the Apo-2 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apo-2 polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. For instance, chemical deglycosylation by exposing the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound can result in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of Apo-2 comprises linking the Apo-2 polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

8. Apo-2 Chimeras

The present invention also provides chimeric molecules comprising Apo-2 fused to another, heterologous polypeptide or amino acid sequence.

In one embodiment, the chimeric molecule comprises a fusion of the Apo-2 with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the Apo-2. The presence of such epitope-tagged forms of the Apo-2 can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Apo-2

to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an α-tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)]. Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein.

Generally, epitope-tagged Apo-2 may be constructed and produced according to the methods described above. Epitope-tagged Apo-2 is also described in the Examples below. Apo-2-tag polypeptide fusions are preferably constructed by fusing the cDNA sequence encoding the Apo-2 portion in-frame to the tag polypeptide DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the Apo-2-tag polypeptide chimeras of the present invention, nucleic acid encoding the Apo-2 will be fused at its 3′ end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5′ fusions are also possible. For example, a polyhistidine sequence of about 5 to about 10 histidine residues may be fused at the N- terminus or the C- terminus and used as a purification handle in affinity chromatography.

Epitope-tagged Apo-2 can be purified by affinity chromatography using the anti-tag antibody. The matrix to which

the affinity antibody is attached may include, for instance, agarose, controlled pore glass or poly(styrenedivinyl)benzene. The epitope-tagged Apo-2 can then be eluted from the affinity column using techniques known in the art.

In another embodiment, the chimeric molecule comprises an Apo-2 polypeptide fused to an immunoglobulin sequence. The chimeric molecule may also comprise a particular domain sequence of Apo-2, such as the extracellular domain sequence of native Apo-2 fused to an immunoglobulin sequence. This includes chimeras in monomeric, homo- or heteromultimeric, and particularly homo- or heterodimeric, or -tetrameric forms; optionally, the chimeras may be in dimeric forms or homodimeric heavy chain forms. Generally, these assembled immunoglobulins will have known unit structures as represented by the following diagrams.

X or A
______________ $C_H$ or $C_L$

X or A
____________Y________ $C_H$ or $C_L$

A
A ______ $C_L$
_______________ $C_H$

A
$V_H$ ______ $C_L$
_______________ $C_H$

$V_L$
A ______ $C_L$
_______________ $C_H$

X
A ______ $C_L$
_______________ $C_H$

A
X ______ $C_L$
_______________ $C_H$

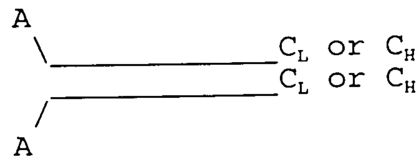
A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four-chain units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in a multimeric form in serum. In the case of multimers, each four chain unit may be the same or different.

The following diagrams depict some exemplary monomer, homo- and heterodimer and homo- and heteromultimer structures. These diagrams are merely illustrative, and the chains of the

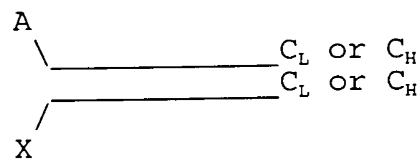
multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins.

monomer: A ____________ $C_L$ or $C_H$

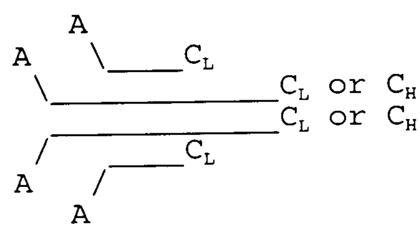
homodimer:



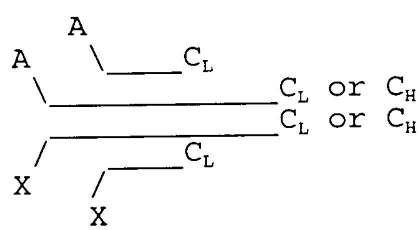

heterodimer:



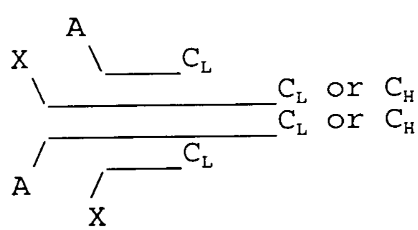

homotetramer:




heterotetramer:




and




In the foregoing diagrams, "A" means an Apo-2 sequence or an Apo-2 sequence fused to a heterologous sequence; X is an additional agent, which may be the same as A or different, a

portion of an immunoglobulin superfamily member such as a variable region or a variable region-like domain, including a native or chimeric immunoglobulin variable region, a toxin such a pseudomonas exotoxin or ricin, or a sequence functionally binding to another protein, such as other cytokines (i.e., IL-1, interferon-$\gamma$) or cell surface molecules (i.e., NGFR, CD40, OX40, Fas antigen, T2 proteins of Shope and myxoma poxviruses), or a polypeptide therapeutic agent not otherwise normally associated with a constant domain; Y is a linker or another receptor sequence; and $V_L$, $V_H$, $C_L$ and $C_H$ represent light or heavy chain variable or constant domains of an immunoglobulin. Structures comprising at least one CRD of an Apo-2 sequence as "A" and another cell-surface protein having a repetitive pattern of CRDs (such as TNFR) as "X" are specifically included.

It will be understood that the above diagrams are merely exemplary of the possible structures of the chimeras of the present invention, and do not encompass all possibilities. For example, there might desirably be several different "A"s, "X"s, or "Y"s in any of these constructs. Also, the heavy or light chain constant domains may be originated from the same or different immunoglobulins. All possible permutations of the illustrated and similar structures are all within the scope of the invention herein.

In general, the chimeric molecules can be constructed in a fashion similar to chimeric antibodies in which a variable domain from an antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; EP 173,494; Munro, Nature, 312:597 (13 December 1984); Neuberger et al., Nature, 312:604-608 (13 December 1984); Sharon et al., Nature, 309:364-367 (24 May 1984); Morrison et al., Proc. Nat'l. Acad. Sci. USA, 81:6851-6855 (1984); Morrison et al., Science, 229:1202-1207 (1985); Boulianne et al., Nature, 312:643-646 (13 December 1984); Capon et al., Nature, 337:525-531 (1989); Traunecker et al., Nature, 339:68-70 (1989).

Alternatively, the chimeric molecules may be constructed

as follows. The DNA including a region encoding the desired sequence, such as an Apo-2 and/or TNFR sequence, is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the immunoglobulin-like domain(s) and at a point at or near the DNA encoding the N-terminal end of the Apo-2 or TNFR polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for TNFR (where the native signal is employed). This DNA fragment then is readily inserted proximal to DNA encoding an immunoglobulin light or heavy chain constant region and, if necessary, the resulting construct tailored by deletional mutagenesis. Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for *in vivo* therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. See for example, Adams et al., Biochemistry, 19:2711-2719 (1980); Gough et al., Biochemistry, 19:2702-2710 (1980); Dolby et al., Proc. Natl. Acad. Sci.. USA, 77:6027-6031 (1980); Rice et al., Proc. Natl. Acad. Sci., 79:7862-7865 (1982); Falkner et al., Nature, 298:286-288 (1982); and Morrison et al., Ann. Rev. Immunol., 2:239-256 (1984).

Further details of how to prepare such fusions are found in publications concerning the preparation of immunoadhesins. Immunoadhesins in general, and CD4-Ig fusion molecules specifically are disclosed in WO 89/02922, published 6 April 1989). Molecules comprising the extracellular portion of CD4, the receptor for human immunodeficiency virus (HIV), linked to IgG heavy chain constant region are known in the art and have been found to have a markedly longer half-life and lower clearance than the soluble extracellular portion of CD4 [Capon et al., supra; Byrn et al., Nature, 344:667 (1990)]. The construction of specific chimeric TNFR-IgG molecules is also described in Ashkenazi et al. Proc. Natl. Acad. Sci., 88:10535-10539 (1991); Lesslauer et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 115 (P 432)]; and Peppel and Beutler, J. Cell. Biochem. Supplement 15F, 1991, p. 118 (P 439)].

B. Therapeutic and Non-therapeutic Uses for Apo-2

Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. This therapy can be accomplished for instance, using *in vivo* or *ex vivo* gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric molecules containing the extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF-$\kappa$B induction by Apo-2L or by another ligand that Apo-2 binds to.

The Apo-2 of the invention also has utility in non-therapeutic applications. Nucleic acid sequences encoding the Apo-2 may be used as a diagnostic for tissue-specific typing. For example, procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding Apo-2 is present in the cell type(s) being evaluated. Apo-2 nucleic acid will also be useful for the preparation of Apo-2 by the recombinant techniques described herein.

The isolated Apo-2 may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of Apo-2 may be prepared. Apo-2 preparations are also useful in generating antibodies, as standards in assays for Apo-2 (*e.g.*, by labeling Apo-2 for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies.

Nucleic acids which encode Apo-2 or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal

(e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding Apo-2 or an appropriate sequence thereof (such as Apo-2-IgG) can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding Apo-2. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for Apo-2 transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding Apo-2 introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding Apo-2. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with excessive apoptosis. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition. In another embodiment, transgenic animals that carry a soluble form of Apo-2 such as the Apo-2 ECD or an immunoglobulin chimera of such form could be constructed to test the effect of chronic neutralization of Apo-2L, a ligand of Apo-2.

Alternatively, non-human homologues of Apo-2 can be used to construct an Apo-2 "knock out" animal which has a defective or altered gene encoding Apo-2 as a result of homologous recombination between the endogenous gene encoding Apo-2 and altered genomic DNA encoding Apo-2 introduced into an embryonic cell of the animal. For example, cDNA encoding Apo-2 can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques. A

portion of the genomic DNA encoding Apo-2 can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., Cell, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the Apo-2 polypeptide, including for example, development of tumors.

C. Anti-Apo-2 Antibody Preparation

The present invention further provides anti-Apo-2 antibodies. Antibodies against Apo-2 may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

1. Polyclonal Antibodies

The Apo-2 antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or

adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is a Apo-2-IgG fusion protein or chimeric molecule (including an Apo-2 ECD-IgG fusion protein). Cells expressing Apo-2 at their surface may also be employed. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. An aggregating agent such as alum may also be employed to enhance the mammal's immune response. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

2. Monoclonal Antibodies

The Apo-2 antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, supra. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is a Apo-2-IgG fusion protein or chimeric molecule. Cells expressing Apo-2 at their surface may also be employed. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or

lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against Apo-2. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are

known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody

of the invention to create a chimeric bivalent antibody.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

*In vitro* methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain ($CH_1$) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain $CH_1$ domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

3. Humanized Antibodies

The Apo-2 antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric

immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Reichmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567),

wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901 (1987)]. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can

be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding [see, WO 94/04679 published 3 March 1994].

Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region ($J_H$) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993)]. Human antibodies can also be produced in phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cote et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cote et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)].

4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have

different specificities [Millstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in

only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [US Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

D. Therapeutic and Non-therapeutic Uses for Apo-2 Antibodies

The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed to activate or stimulate apoptosis in cancer cells. Alternatively, antagonistic antibodies may be used to block excessive apoptosis (for instance in neurodegenerative disease) or to block potential autoimmune/inflammatory effects of Apo-2 resulting from NF-$\kappa$B activation.

Apo-2 antibodies may further be used in diagnostic assays for Apo-2, *e.g.*, detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled

with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as $^{3}H$, $^{14}C$, $^{32}P$, $^{35}S$, or $^{125}I$, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

Apo-2 antibodies also are useful for the affinity purification of Apo-2 from recombinant cell culture or natural sources. In this process, the antibodies against Apo-2 are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-2 to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the Apo-2, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the Apo-2 from the antibody.

E. Kits Containing Apo-2 or Apo-2 Antibodies

In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active agent in the composition is Apo-2 or an Apo-2 antibody. The label

on the container indicates that the composition is used for a specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

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The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

## EXAMPLES

All restriction enzymes referred to in the examples were purchased from New England Biolabs and used according to manufacturer's instructions. All other commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Rockville, Maryland.

## EXAMPLE 1

### Isolation of cDNA clones Encoding Human Apo-2

Expressed sequence tag (EST) DNA databases (LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA) were searched and an EST was identified which showed homology to the death domain of the Apo-3 receptor [Marsters et al., Curr. Biol., 6:750 (1996)]. Human pancreas and kidney lgt10 bacteriophage cDNA libraries (both purchased from Clontech) were ligated into pRK5 vectors as follows.

Reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (3 ml); pRK5, Xhol, Notl digested vector, 0.5 mg, 1 ml); cDNA (5 ml) and distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) were added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was removed, collected and diluted into 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, and the pellet resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The DNA pellet was then dried in a speedvac and eluted into distilled water (3 ml) for use in the subsequent procedure.

The ligated cDNA/pRK5 vector DNA prepared previously was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then electroporated as per the manufacturers recommendation. Subsequently SOC media (1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C) to allow the colonies to grow. Positive colonies were then scraped off and the DNA isolated from the bacterial pellet using standard CsCl-gradient protocols.

An enriched 5'-cDNA library was then constructed to obtain a bias of cDNA fragments which preferentially represents the 5' ends of cDNA's contained within the library. 10 mg of the pooled isolated full-length library plasmid DNA (41 ml) was combined with Not 1 restriction buffer (New England Biolabs, 5 ml) and Not 1 (New England Biolabs, 4 ml) and incubated at 37°C for one hour. The reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml), the aqueous phase removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was then removed, the pellet dried in a speedvac and resuspended in

distilled water (10 ml).

The following reagents were brought together and incubated at 37°C for 2 hours: distilled water (3 ml); linearized DNA library (1 mg, 1 ml); Ribonucleotide mix (Invitrogen, 10 ml); transcription buffer (Invitrogen, 2 ml) and Sp6 enzyme mix. The reaction was then extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml) and the aqueous phase was removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml) and centrifuged for 20 minutes at 14,000 x g. The pellet was then decanted and resuspended in 70% ethanol (0.5 ml), centrifuged again for 2 minutes at 14,000 x g, decanted, dried in a speedvac and resuspended into distilled water (10 ml).

The following reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (Life Tech., 3 ml); pRK5 Cla-Sal digested vector, 0.5 mg, 1 ml); cDNA (5 ml); distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) was added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 100 ml). The aqueous phase was removed, collected and diluted by 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml) and centrifuged for 20 minutes at 14,000 x g. The DNA pellet was decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was removed and the residue pellet was dried in a speedvac and resuspended in distilled water (3ml). The ligated cDNA/pSST-amy.1 vector DNA was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then electroporated as recommended by the manufacturer. Subsequently, SOC media (Life Tech., 1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C). Positive colonies were scraped off the plates and the DNA was isolated from the bacterial pellet using standard protocols, e.g. CsCl-gradient.

The cDNA libraries were screened by hybridization with a

synthetic oligonucleotide probe: GGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGGCA GCGGG (SEQ ID NO:3) based on the EST.

Three cDNA clones were sequenced in entirety. The overlapping coding regions of the cDNAs were identical except for codon 410 (using the numbering system for Fig. 1); this position encoded a leucine residue (TTG) in both pancreatic cDNAs, and a methionine residue (ATG) in the kidney cDNA, possibly due to polymorphism.

The entire nucleotide sequence of Apo-2 is shown in Figure 1 (SEQ ID NO:2). Clone 27868 (also referred to as pRK5-Apo-2 deposited as ATCC _____, as indicated below) contains a single open reading frame with an apparent translational initiation site at nucleotide positions 140-142 [Kozak et al., supra] and ending at the stop codon found at nucleotide positions 1373-1375 (Fig. 1; SEQ ID NO:2). The predicted polypeptide precursor is 411 amino acids long, a type I transmembrane protein, and has a calculated molecular weight of approximately 45 kDa. Hydropathy analysis (not shown) suggested the presence of a signal sequence (residues 1-53), followed by an extracellular domain (residues 54-182), a transmembrane domain (residues 183-208), and an intracellular domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino acid sequence analysis of Apo-2-IgG expressed in 293 cells showed that the mature polypeptide starts at amino acid residue 54, indicating that the actual signal sequence comprises residues 1-53.

TNF receptor family proteins are typically characterized by the presence of multiple (usually four) cysteine-rich domains in their extracellular regions -- each cysteine-rich domain being approximately 45 amino acids long and containing approximately 6, regularly spaced, cysteine residues. Based on the crystal structure of the type 1 TNF receptor, the cysteines in each domain typically form three disulfide bonds in which usually cysteines 1 and 2, 3 and 5, and 4 and 6 are paired together. Like DR4, Apo-2 contains two extracellular cysteine-rich pseudorepeats (Fig. 2A), whereas other identified mammalian TNFR family members contain

three or more such domains [Smith et al., Cell, 76:959 (1994)].

The cytoplasmic region of Apo-2 contains a death domain (amino acid residues 324-391 shown in Fig. 1; see also Fig. 2A) which shows significantly more amino acid sequence identity to the death domain of DR4 (64%) than to the death domain of TNFR1 (30%); CD95 (19%); or Apo-3/DR3 (29%) (Fig. 2B). Four out of six death domain amino acids that are required for signaling by TNFR1 [Tartaglia et al., supra] are conserved in Apo-2 while the other two residues are semi-conserved (see Fig. 2B).

Based on an alignment analysis (using the ALIGN™ computer program) of the full-length sequence, Apo-2 shows more sequence identity to DR4 (55%) than to other apoptosis-linked receptors, such as TNFR1 (19%); CD95 (17%); or Apo-3 (also referred to as DR3, WSL-1 or TRAMP) (29%).

## EXAMPLE 2

### A. Expression of Apo-2 ECD

A soluble extracellular domain (ECD) fusion construct was prepared. An Apo-2 ECD (amino acid residues 1-184 shown in Figure 1) was obtained by PCR and fused to a C-terminal Flag epitope tag (Sigma). (The Apo-2 ECD construct included residues 183 and 184 shown in Figure 1 to provide flexibility at the junction, even though residues 183 and 184 are predicted to be in the transmembrane region). The Flag epitope-tagged molecule was then inserted into pRK5, and expressed by transient transfection into human 293 cells (ATCC CRL 1573).

After a 48 hour incubation, the cell supernatants were collected and either used directly for co-precipitation studies (see Example 3) or subjected to purification of the Apo-2 ECD-Flag by affinity chromatography on anti-Flag agarose beads, according to manufacturer's instructions (Sigma).

### B. Expression of Apo-2 ECD as an Immunoadhesin

A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the

hinge and Fc region of human immunoglobulin $G_1$ heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., supra.

## EXAMPLE 3

### Immunoprecipitation Assay Showing Binding Interaction Between Apo-2 and Apo-2 Ligand

To determine whether Apo-2 and Apo-2L interact or associate with each other, supernatants from mock-transfected 293 cells or from 293 cells transfected with Apo-2 ECD-Flag (described in Example 2 above) (5 ml) were incubated with 5 μg poly-histidine-tagged soluble Apo-2L [Pitti et al., supra] for 30 minutes at room temperature and then analyzed for complex formation by a co-precipitation assay.

The samples were subjected to immunoprecipitation using 25 μl anti-Flag conjugated agarose beads (Sigma) or Nickel-conjugated agarose beads (Qiagen). After a 1.5 hour incubation at 4° C, the beads were spun down and washed four times in phosphate buffered saline (PBS). By using anti-Flag agarose, the Apo-2L was precipitated through the Flag-tagged Apo-2 ECD; by using Nickel-agarose, the Apo-2 ECD was precipitated through the His-tagged Apo-2L. The precipitated proteins were released by boiling the beads for 5 minutes in SDS-PAGE buffer, resolved by electrophoresis on 12% polyacrylamide gels, and then detected by immunoblot with anti-Apo-2L or anti-Flag antibody (2 μg/ml) as described in Marsters et al., J. Biol. Chem., (1997).

The results, shown in Figure 3, indicate that the Apo-2 ECD and Apo-2L can associate with each other.

The binding interaction was further analyzed by purifying Apo-2 ECD from the transfected 293 cell supernatants with anti-Flag beads (see Example 2) and then analyzing the samples on a BIACORE™ instrument. The BIACORE™ analysis indicated a dissociation

constant ($K_d$) of about 1 nM. BIACORE™ analysis also showed that the Apo-2 ECD is not capable of binding other apoptosis-inducing TNF family members, namely, TNF-alpha (Genentech, Inc., Pennica et al., Nature, 312:712 (1984), lymphotoxin-alpha (Genentech, Inc.), or Fas/Apo-1 ligand (Alexis Biochemicals). The data thus shows that Apo-2 is a specific receptor for Apo-2L.

## EXAMPLE 4

### Induction of Apoptosis by Apo-2

Because death domains can function as oligomerization interfaces, over-expression of receptors that contain death domains may lead to activation of signaling in the absence of ligand [Frazer et al., supra, Nagata et al., supra]. To determine whether Apo-2 was capable of inducing cell death, human 293 cells or HeLa cells (ATCC CCL 2.2) were transiently transfected by calcium phosphate precipitation (293 cells) or electroporation (HeLa cells) with a pRK5 vector or pRK5-based plasmids encoding Apo-2 and/or CrmA. When applicable, the total amount of plasmid DNA was adjusted by adding vector DNA. Apoptosis was assessed 24 hours after transfection by morphology (Fig. 4A); DNA fragmentation (Fig. 4B); or by FACS analysis of phosphatydilserine exposure (Fig. 4C) as described in Marsters et al., Curr. Biol., 6:1669 (1996). As shown in Figs. 4A and 4B, the Apo-2 transfected 293 cells underwent marked apoptosis.

For samples assayed by FACS, the HeLa cells were co-transfected with pRK5-CD4 as a marker for transfection and apoptosis was determined in CD4-expressing cells; FADD was co-transfected with the Apo-2 plasmid; the data are means ± SEM of at least three experiments, as described in Marsters et al., Curr. Biol., 6:1669 (1996). The caspase inhibitors, DEVD-fmk (Enzyme Systems) or z-VAD-fmk (Research Biochemicals Intl.) were added at 200 $\mu$M at the time of transfection. As shown in Fig. 4C, the caspase inhibitors CrmA, DEVD-fmk, and z-VAD-fmk blocked apoptosis induction by Apo-2, indicating the involvement of Ced-3-like proteases in this response.

FADD is an adaptor protein that mediates apoptosis activation by CD95, TNFR1, and Apo-3/DR3 [Nagata et al., supra], but does not appear necessary for apoptosis induction by Apo-2L [Marsters et al., supra] or by DR4 [Pan et al., supra]. A dominant-negative mutant form of FADD, which blocks apoptosis induction by CD95, TNFR1, or Apo-3/DR3 [Frazer et al., supra; Nagata et al., supra; Chinnayian et al., supra] did not inhibit apoptosis induction by Apo-2 when co-transfected into HeLa cells with Apo-2 (Fig. 4C). These results suggest that Apo-2 signals apoptosis independently of FADD. Consistent with this conclusion, a glutathione-S-transferase fusion protein containing the Apo-2 cytoplasmic region did not bind to *in vitro* transcribed and translated FADD (data not shown).

## EXAMPLE 5

### Inhibition of Apo-2L Activity by Soluble Apo-2 ECD

Soluble Apo-2L (0.5 μg/ml, prepared as described in Pitti et al., supra) was pre-incubated for 1 hour at room temperature with PBS buffer or affinity-purified Apo-2 ECD (5 μg/ml) together with anti-Flag antibody (Sigma) (1 μg/ml) and added to HeLa cells. After a 5 hour incubation, the cells were analyzed for apoptosis by FACS (as above) (Fig. 4D).

Apo-2L induced marked apoptosis in HeLa cells, and the soluble Apo-2 ECD was capable of blocking Apo-2L action (Fig. 4D), confirming a specific interaction between Apo-2L and Apo-2. Similar results were obtained with the Apo-2 ECD immunoadhesin (Fig. 4D). Dose-response analysis showed half-maximal inhibition at approximately 0.3 nM Apo-2 immunoadhesin (Fig. 4E).

## EXAMPLE 6

### Activation of NF-κB by Apo-2

An assay was conducted to determine whether Apo-2 activates NF-κB.

HeLa cells were transfected with pRK5 expression plasmids encoding full-length native sequence Apo-2, DR4 or Apo-3 and

harvested 24 hours after transfection. Nuclear extracts were prepared and 1 $\mu g$ of nuclear protein was reacted with a $^{32}P$-labelled NF-$\kappa$B-specific synthetic oligonucleotide probe ATCAGGGACTTTCCGCTGGGGACTTTCCG (SEQ ID NO:4) [see, also, MacKay et al., J. Immunol., 153:5274-5284 (1994)], alone or together with a 50-fold excess of unlabelled probe, or with an irrelevant $^{32}P$-labelled synthetic oligonucleotide AGGATGGGAAGTGTGTGATATATCCTTGAT (SEQ ID NO:5). In some samples, antibody to p65/RelA subunits of NF-$\kappa$B (1 $\mu g/ml$; Santa Cruz Biotechnology) was added. DNA binding was analyzed by an electrophoretic mobility shift assay as described by Hsu et al., supra; Marsters et al., supra, and MacKay et al., supra.

The results are shown in Fig. 5. As shown in Fig. 5A, upon transfection into HeLa cells, both Apo-2 and DR4 induced significant NF-$\kappa$B activation as measured by the electrophoretic mobility shift assay; the level of activation was comparable to activation observed for Apo-3/DR3. Antibody to the p65/RelA subunit of NF-$\kappa$B inhibited the mobility of the NF-$\kappa$B probe, implicating p65 in the response to all 3 receptors.

An assay was also conducted to determine if Apo-2L itself can regulate NF-$\kappa$B activity. HeLa cells or MCF7 cells (human breast adenocarcinoma cell line, ATCC HTB 22) were treated with PBS buffer, soluble Apo-2L (Pitti et al., supra) or TNF-alpha (Genentech, Inc., see Pennica et al., Nature, 312:721 (1984)) (1 $\mu g/ml$) and assayed for NF-$\kappa$B activity as above. The results are shown in Fig. 5B. The Apo-2L induced a significant NF-$\kappa$B activation in the treated HeLa cells but not in the treated MCF7 cells; the TNF-alpha induced a more pronounced activation in both cell lines. Several studies have disclosed that NF-$\kappa$B activation by TNF can protect cells against TNF-induced apoptosis [Nagata, supra].

The effects of a NF-$\kappa$B inhibitor, ALLN (N-acetyl-Leu-Leu-norleucinal) and a transcription inhibitor, cyclohexamide, were also tested. The HeLa cells (plated in 6-well dishes) were preincubated with PBS buffer, ALLN (Calbiochem) (40 $\mu g/ml$) or

cyclohexamide (Sigma) (50 μg/ml) for 1 hour before addition of Apo-2L (1 μg/ml). After a 5 hour incubation, apoptosis was analyzed by FACS (see Fig. 5C).

The results are shown in Fig. 5C. Both ALLN and cyclohexamide increased the level of Apo-2L-induced apoptosis in the HeLa cells. The data indicates that Apo-2L can induce protective NF-κB-dependent genes. The data also indicates that Apo-2L is capable of activating NF-κB in certain cell lines and that both Apo-2 and DR4 may mediate that function.

## EXAMPLE 7

## Northern Blot Analysis

Expression of Apo-2 mRNA in human tissues was examined by Northern blot analysis. Human RNA blots were hybridized to a 4.6 kilobase $^{32}P$-labelled DNA probe based on the full length Apo-2 cDNA; the probe was generated by digesting the pRK5-Apo-2 plasmid with EcoRI. Human fetal RNA blot MTN (Clontech) and human adult RNA blot MTN-II (Clontech) were incubated with the DNA probes. Blots were incubated with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after overnight exposure.

As shown in Fig. 6, a predominant mRNA transcript of approximately 4.6kb was detected in multiple tissues. Expression was relatively high in fetal and adult liver and lung, and in adult ovary and peripheral blood leukocytes (PBL), while no mRNA expression was detected in fetal and adult brain. Intermediate levels of expression were seen in adult colon, small intestine, testis, prostate, thymus, pancreas, lidney, skeletal muscle, placenta, and heart. Several adult tissues that express Apo-2, e.g., PBL, ovary, and spleen, have been shown previously to express DR4 [Pan et al., supra], however, the relative levels of expression of each receptor mRNA appear to be different.

## EXAMPLE 8

### Chromosomal Localization of the Apo-2 gene

Chromosomal localization of the human Apo-2 gene was examined by radiation hybrid (RH) panel analysis. RH mapping was performed by PCR using a human-mouse cell radiation hybrid panel (Research Genetics) and primers based on the coding region of the Apo-2 cDNA [Gelb et al., Hum. Genet., 98:141 (1996)]. Analysis of the PCR data using the Stanford Human Genome Center Database indicates that Apo-2 is linked to the marker D8S481, with an LOD of 11.05; D8S481 is linked in turn to D8S2055, which maps to human chromosome 8p21. A similar analysis of DR4 showed that DR4 is linked to the marker D8S2127 (with an LOD of 13.00), which maps also to human chromosome 8p21.

To Applicants' present knowledge, to date, no other member of the TNFR gene family has been located to chromosome 8.

* * * * *

Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

| Material | ATCC Dep. No. | Deposit Date |
| --- | --- | --- |
| pRK5-Apo-2 | ____________ | ___________ |

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the

invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Ashkenazi, Avi J.

(ii) TITLE OF INVENTION: Apo-2 RECEPTOR

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 460 Point San Bruno Blvd
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 15-May-1997
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Marschang, Diane L.
(B) REGISTRATION NUMBER: 35,600
(C) REFERENCE/DOCKET NUMBER: P1101

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 415/225-5416
(B) TELEFAX: 415/952-9881
(C) TELEX: 910/371-7168

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 411 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro
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Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val
                35                  40                  45

Leu Leu Leu Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp
                50                  55                  60

Leu Ala Pro Gln Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser
                65                  70                  75

Pro Ser Glu Gly Leu Cys Pro Pro Gly His His Ile Ser Glu Asp
                80                  85                  90

Gly Arg Asp Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr
                95                 100                 105

His Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys Asp
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110 115 120

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Val Cys Gln Cys Glu Glu Gly Thr Phe Arg Glu Glu Asp Ser Pro
140 145 150

Glu Met Cys Arg Lys Cys Arg Thr Gly Cys Pro Arg Gly Met Val
155 160 165

Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile Glu Cys Val His
170 175 180

Lys Glu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val
185 190 195

Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys
200 205 210

Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp
215 220 225

Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp
230 235 240

Asn Val Leu Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val
245 250 255

Pro Glu Gln Glu Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly
260 265 270

Val Asn Met Leu Ser Pro Gly Glu Ser Glu His Leu Leu Glu Pro
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Ala Glu Ala Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala
290 295 300

Asn Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp Asp
305 310 315

Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro Leu Met Arg
320 325 330

Lys Leu Gly Leu Met Asp Asn Glu Ile Lys Val Ala Lys Ala Glu
335 340 345

Ala Ala Gly His Arg Asp Thr Leu Tyr Thr Met Leu Ile Lys Trp
350 355 360

Val Asn Lys Thr Gly Arg Asp Ala Ser Val His Thr Leu Leu Asp
365 370 375

Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu
380 385 390

Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn
395 400 405

Ala Asp Ser Ala Xaa Ser
410 411

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1799 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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                                            Met Glu
                                             1

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                 20                  25

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Arg Pro Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val
     30                  35                  40

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Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln Gln Arg Ala
 55                  60                  65

GCC CCA CAA CAA AAG AGG TCC AGC CCC TCA GAG GGA TTG 379
Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu
         70                  75                  80
```

```
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                85                  90

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Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His
     95                 100                 105

TGG AAT GAC CTC CTT TTC TGC TTG CGC TGC ACC AGG TGT 496
Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys
            110                 115

GAT TCA GGT GAA GTG GAG CTA AGT CCC TGC ACC ACG ACC 535
Asp Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr
120                 125                 130

AGA AAC ACA GTG TGT CAG TGC GAA GAA GGC ACC TTC CGG 574
Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe Arg
        135                 140                 145

GAA GAA GAT TCT CCT GAG ATG TGC CGG AAG TGC CGC ACA 613
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                150                 155

GGG TGT CCC AGA GGG ATG GTC AAG GTC GGT GAT TGT ACA 652
Gly Cys Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr
    160                 165                 170

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185                 190                 195
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215 220

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GGG GCT GAG GAC AAT GTC CTC AAT GAG ATC GTG AGT ATC 886
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TTG CAG CCC ACC CAG GTC CCT GAG CAG GAA ATG GAA GTC 925
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250 255 260

CAG GAG CCA GCA GAG CCA ACA GGT GTC AAC ATG TTG TCC 964
Gln Glu Pro Ala Glu Pro Thr Gly Val Asn Met Leu Ser
265 270 275

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280 285

GAA AGG TCT CAG AGG AGG AGG CTG CTG GTT CCA GCA AAT 1042
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290 295 300

GAA GGT GAT CCC ACT GAG ACT CTG AGA CAG TGC TTC GAT 1081
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305 310

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315 320 325

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330 335 340

GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG 1198
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345 350

TAC ACG ATG CTG ATA AAG TGG GTC AAC AAA ACC GGG CGA 1237
Tyr Thr Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg
355 360 365

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395 400 405

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Ala Asp Ser Ala Xaa Ser
410 411

CCTTCCCTGG TTTACCTTTT TTCTGGAAAA AGCCCAACTG GACTCCAGTC 1450

AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC 1500

CCATCCAACA TCACCCAGTG GATGGAACAT CCTGTAACTT TTCACTGCAC 1550

TTGGCATTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT 1600

GTCTGGATCA TTCCGTTTGT GCGTACTTTG AGATTTGGTT TGGGATGTCA 1650

TTGTTTTCAC AGCACTTTTT TATCCTAATG TAAATGCTTT ATTTATTTAT 1700

TTGGGCTACA TTGTAAGATC CATCTACAAA AAAAAAAAAA AAAAAAAAAG 1750

GGCGGCCGCG ACTCTAGAGT CGACCTGCAG AAGCTTGGCC GCCATGGCC 1799

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGAGCCGCT CATGAGGAAG TTGGGCCTCA TGGACAATGA GATAAAGGTG 50

GCTAAAGCTG AGGCAGCGGG 70

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATCAGGGACT TTCCGCTGGG GACTTTCCG 29

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGGATGGGAA GTGTGTGATA TATCCTTGAT 30

WHAT IS CLAIMED IS:

1. A method of modulating apoptosis in mammalian cells comprising exposing mammalian cells to Apo-2 polypeptide, said Apo-2 polypeptide having at least about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising amino acid residues 1 to 411 of SEQ ID NO:1.

2. A method of modulating apoptosis in mammalian cells comprising exposing mammalian cells to an extracellular domain sequence of Apo-2 polypeptide comprising amino acid residues 54 to 182 of SEQ ID NO:1.

## Abstract of the Disclosure

Novel polypeptides, designated Apo-2, which are capable of modulating apoptosis are provided. Compositions including Apo-2 chimeras, nucleic acid encoding Apo-2, and antibodies to Apo-2 are also provided.

Fig. 1

```
   1 CCCACGCGTC CGCATAAATC AGCACGCGGC CGGAGAACCC CGCAATCTCT GCGCCCACAA AATACACCGA CGATGCCCGA TCTACTTTAA GGGCTGAAAC
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 101 CCACGGGCCT GAGAGACTAT AAGAGCGTTC CCTACCGCCA TGGAACAACG GGGACAGAAC GCCCCGGCCG CTTCGGGGGC CCGGAAAAGG CACGGCCCAG
     GGTGCCCGGA CTCTCTGATA TTCTCGCAAG GGATGGCGGT ACCTTGTTGC CCCTGTCTTG CGGGGCCGGC GAAGCCCCCG GGCCTTTTCC GTGCCGGGTC
   1                                            M etGluGlnAr gGlyGlnAsn AlaProAlaA laSerGlyAl aArgLysArg HisGlyProGly

 201 GACCCAGGGA GGCGCGGGGA GCCAGGCCTG GGCTCCGGGT CCCCAAGACC CTTGTGCTCG TTGTCGCCGC GGTCCTGCTG TTGGTCTCAG CTGAGTCTGC
     CTGGGTCCCT CCGCGCCCCT CGGTCCGGAC CCGAGGCCCA GGGGTTCTGG GAACACGAGC AACAGCGGCG CCAGGACGAC AACCAGAGTC GACTCAGACG
  22   ProArgGl uAlaArgGly AlaArgProG lyLeuArgVa lProLysThr LeuValLeuV alValAlaAl aValLeuLeu LeuValSerA laGluSerAla

 301 TCTGATCACC CAACAAGACC TAGCTCCCCA GCAGAGAGCG GCCCCACAAC AAAAGAGGTC CAGCCCCTCA GAGGGATTGT GTCCACCTGG ACACCATATC
     AGACTAGTGG GTTGTTCTGG ATCGAGGGGT CGTCTCTCGC CGGGGTGTTG TTTTCTCCAG GTCGGGGAGT CTCCCTAACA CAGGTGGACC TGTGGTATAG
  55  LeuIleThr GlnGlnAspL euAlaProGl nGlnArgAla AlaProGlnG lnLysArgSe rSerProSer GluGlyLeuC ysProProGl yHisHisIle

 401 TCAGAAGACG GTAGAGATTG CATCTCCTGC AAATATGGAC AGGACTATAG CACTCACTGG AATGACCTCC TTTTCTGCTT GCGCTGCACC AGGTGTGATT
     AGTCTTCTGC CATCTCTAAC GTAGAGGACG TTTATACCTG TCCTGATATC GTGAGTGACC TTACTGGAGG AAAAGACGAA CGCGACGTGG TCCACACTAA
  88 SerGluAspG lyArgAspCy sIleSerCys LysTyrGlyG lnAspTyrSe rThrHisTrp AsnAspLeuL euPheCysLe uArgCysThr ArgCysAspSer

 501 CAGGTGAAGT GGAGCTAAGT CCCTGCACCA CGACCAGAAA CACAGTGTGT CAGTGCGAAG AAGGCACCTT CCGGGAAGAA GATTCTCCTG AGATGTGCCG
     GTCCACTTCA CCTCGATTCA GGGACGTGGT GCTGGTCTTT GTGTCACACA GTCACGCTTC TTCCGTGGAA GGCCCTTCTT CTAAGAGGAC TCTACACGGC
 122   GlyGluVa lGluLeuSer ProCysThrT hrThrArgAs nThrValCys GlnCysGluG luGlyThrPh eArgGluGlu AspSerProG luMetCysArg

 601 GAAGTGCCGC ACAGGGTGTC CCAGAGGGAT GGTCAAGGTC GGTGATTGTA CACCCTGGAG TGACATCGAA TGTGTCCACA AAGAATCAGG CATCATCATA
     CTTCACGGCG TGTCCCACAG GGTCTCCCTA CCAGTTCCAG CCACTAACAT GTGGGACCTC ACTGTAGCTT ACACAGGTGT TTCTTAGTCC GTAGTAGTAT
 155  LysCysArg ThrGlyCysP roArgGlyMe tValLysVal GlyAspCysT hrProTrpSe rAspIleGlu CysValHisL ysGluSerGl yIleIleIle

 701 GGAGTCACAG TTGCAGCCGT AGTCTTGATT GTGGCTGTGT TTGTTTGCAA GTCTTTACTG TGGAAGAAAG TCCTTCCTTA CCTGAAAGGC ATCTGCTCAG
     CCTCAGTGTC AACGTCGGCA TCAGAACTAA CACCGACACA AACAAACGTT CAGAAATGAC ACCTTCTTTC AGGAAGGAAT GGACTTTCCG TAGACGAGTC
 188 GlyValThrV alAlaAlaVa lValLeuIle ValAlaValP heValCysLy sSerLeuLeu TrpLysLysV alLeuProTy rLeuLysGly IleCysSerGly

 801 GTGGTGGTGG GGACCCTGAG CGTGTGGACA GAAGCTCACA ACGACCTGGG GCTGAGGACA ATGTCCTCAA TGAGATCGTG AGTATCTTGC AGCCCACCCA
     CACCACCACC CCTGGGACTC GCACACCTGT CTTCGAGTGT TGCTGGACCC CGACTCCTGT TACAGGAGTT ACTCTAGCAC TCATAGAACG TCGGGTGGGT
 222   GlyGlyGl yAspProGlu ArgValAspA rgSerSerGl nArgProGly AlaGluAspA snValLeuAs nGluIleVal SerIleLeuG lnProThrGln

 901 GGTCCCTGAG CAGGAAATGG AAGTCCAGGA GCCAGCAGAG CCAACAGGTG TCAACATGTT GTCCCCCGGG GAGTCAGAGC ATCTGCTGGA ACCGGCAGAA
     CCAGGGACTC GTCCTTTACC TTCAGGTCCT CGGTCGTCTC GGTTGTCCAC AGTTGTACAA CAGGGGGCCC CTCAGTCTCG TAGACGACCT TGGCCGTCTT
 255  ValProGlu GlnGluMetG luValGlnGl uProAlaGlu ProThrGlyV alAsnMetLe uSerProGly GluSerGluH isLeuLeuGl uProAlaGlu

1001 GCTGAAAGGT CTCAGAGGAG GAGGCTGCTG GTTCCAGCAA ATGAAGGTGA TCCCACTGAG ACTCTGAGAC AGTGCTTCGA TGACTTTGCA GACTTGGTGC
     CGACTTTCCA GAGTCTCCTC CTCCGACGAC CAAGGTCGTT TACTTCCACT AGGGTGACTC TGAGACTCTG TCACGAAGCT ACTGAAACGT CTGAACCACG
 288 AlaGluArgS erGlnArgAr gArgLeuLeu ValProAlaA snGluGlyAs pProThrGlu ThrLeuArgG lnCysPheAs pAspPheAla AspLeuValPro
```

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1101 CCTTTGACTC CTGGGAGCCG CTCATGAGGA AGTTGGGCCT CATGGACAAT GAGATAAAGG TGGCTAAAGC TGAGGCAGCG GGCCACAGGG ACACCTTGTA
     GGAAACTGAG GACCCTCGGC GAGTACTCCT TCAACCCGGA GTACCTGTTA CTCTATTTCC ACCGATTTCG ACTCCGTCGC CCGGTGTCCC TGTGGAACAT
 322   PheAspSe rTrpGluPro LeuMetArgL ysLeuGlyLe uMetAspAsn GluIleLysV alAlaLysAl aGluAlaAla GlyHisArgA spThrLeuTyr

1201 CACGATGCTG ATAAAGTGGG TCAACAAAAC CGGGCGAGAT GCCTCTGTCC ACACCCTGCT GGATGCCTTG GAGACGCTGG GAGAGAGACT TGCCAAGCAG
     GTGCTACGAC TATTTCACCC AGTTGTTTTG GCCCGCTCTA CGGAGACAGG TGTGGGACGA CCTACGGAAC CTCTGCGACC CTCTCTCTGA ACGGTTCGTC
 355  ThrMetLeu IleLysTrpV alAsnLysTh rGlyArgAsp AlaSerValH isThrLeuLe uAspAlaLeu GluThrLeuG lyGluArgLe uAlaLysGln

1301 AAGATTGAGG ACCACTTGTT GAGCTCTGGA AAGTTCATGT ATCTAGAAGG TAATGCAGAC TCTGCCWTGT CCTAAGTGTG ATTCTCTTCA GGAAGTGAGA
     TTCTAACTCC TGGTGAACAA CTCGAGACCT TTCAAGTACA TAGATCTTCC ATTACGTCTG AGACGGAACA GGATTCACAC TAAGAGAAGT CCTTCACTCT
 388 LysIleGluA spHisLeuLe uSerSerGly LysPheMetT yrLeuGluGl yAsnAlaAsp SerAlaXaaS erOC*

1401 CCTTCCCTGG TTTACCTTTT TTCTGGAAAA AGCCCAACTG GACTCCAGTC AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC
     GGAAGGGACC AAATGGAAAA AAGACCTTTT TCGGGTTGAC CTGAGGTCAG TCATCCTTTC ACGGTGTTAA CAGTGTACTG GCCATGACCT TCTTTGAGAG

1501 CCATCCAACA TCACCCAGTG GATGGAACAT CCTGTAACTT TTCACTGCAC TTGGCATTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT
     GGTAGGTTGT AGTGGGTCAC CTACCTTGTA GGACATTGAA AAGTGACGTG AACCGTAATA AAAATATTCG ACTTACACTA TTATTCCTGT GATACCTTTA

1601 GTCTGGATCA TTCCGTTTGT GCGTACTTTG AGATTTGGTT TGGGATGTCA TTGTTTTCAC AGCACTTTTT TATCCTAATG TAAATGCTTT ATTTATTTAT
     CAGACCTAGT AAGGCAAACA CGCATGAAAC TCTAAACCAA ACCCTACAGT AACAAAAGTG TCGTGAAAAA ATAGGATTAC ATTTACGAAA TAAATAAATA

1701 TTGGGCTACA TTGTAAGATC CATCTACAAA AAAAAAAAAA AAAAAAAAAG GGCGGCCGCG ACTCTAGAGT CGACCTGCAG AAGCTTGGCC GCCATGGCC
     AACCCGATGT AACATTCTAG GTAGATGTTT TTTTTTTTTT TTTTTTTTTC CCGCCGGCGC TGAGATCTCA GCTGGACGTC TTCGAACCGG CGGTACCGG
```

Fig. 1 (cont.)

FIG. 2A

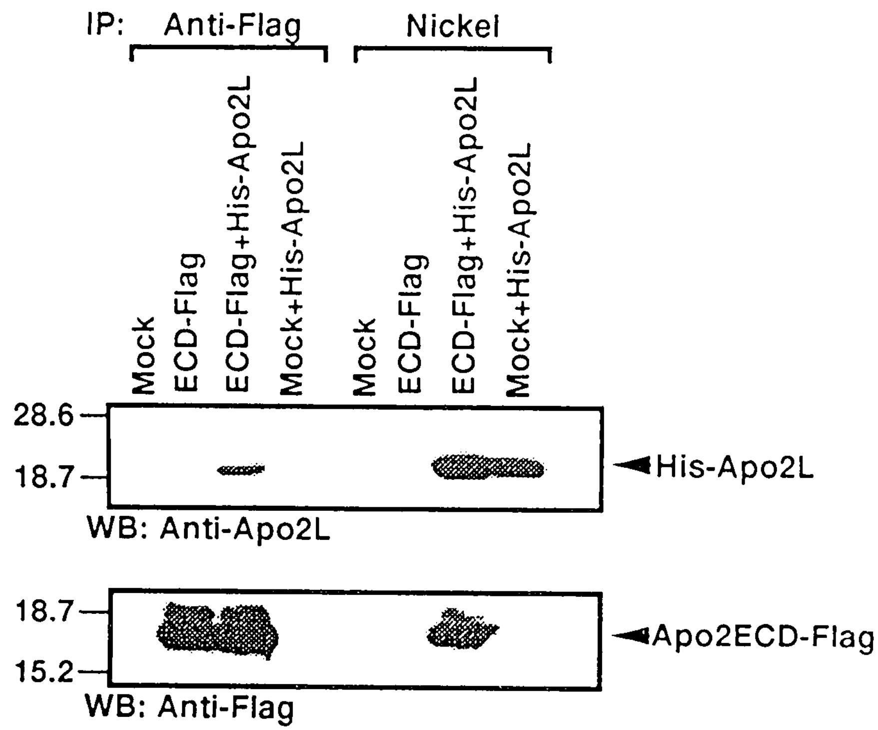
1 MEQRGQNAPAASGARKRHGPGPREARGARPGLRVPKTLVLVVAAVLLLVSAESALITQQD
61 LAPQQRAAPQQKRSSPSEGLCPPGHHISEDGRDCISCKYGQDYSTHWNDLLFCLRCTRCD
121 SGEVELSPCTTTRNTVCQCEEGTFREEDSPEMCRKCRTGCPRGMVKVGDCTPWSDIECVH
181 KESGIIIGVTVAAVVLIVAVFVCKSLLWKKVLPYLKGICSGGGGDPERVDRSSQRPGAED
241 NVLNEIVSILQPTQVPEQEMEVQEPAEPTGVNMLSPGESEHLLEPAEAERSQRRRLLVPA
301 NEGDPTETLRQCFDDFADLVPFDSWEPLMRKLGLMDNEIKVAKAEAAGHRDTLYTMLIKW
361 VNKTGRDASVHTLLDALETLGERLAKQKIEDHLLSSGKFMYLEGNADSALS

Fig. 2B

| | |
|---|---|
| Apo2 | F A D L V P F D S W E P L M R K L G L M D N E I K V A K A E A A - - G H R D T L |
| DR4 | F A N I V P F D S W D Q L M R Q L D L T K N E I D V V R A G T A - - G P G D A L |
| Apo3/DR3 | V M D A V P A R R W K E F V R T L G L R E A E I E A V E V E I G R - - F R D Q Q |
| TNFR1 | V V E N V P P L R W K E F V R R L G L S D H E I D R L E L Q N G R - C L R E A Q |
| Fas/Apo1 | I A G V M T L S Q V K G F V R K N G V N E A K I D E I K N D N V Q D T A E Q K V |

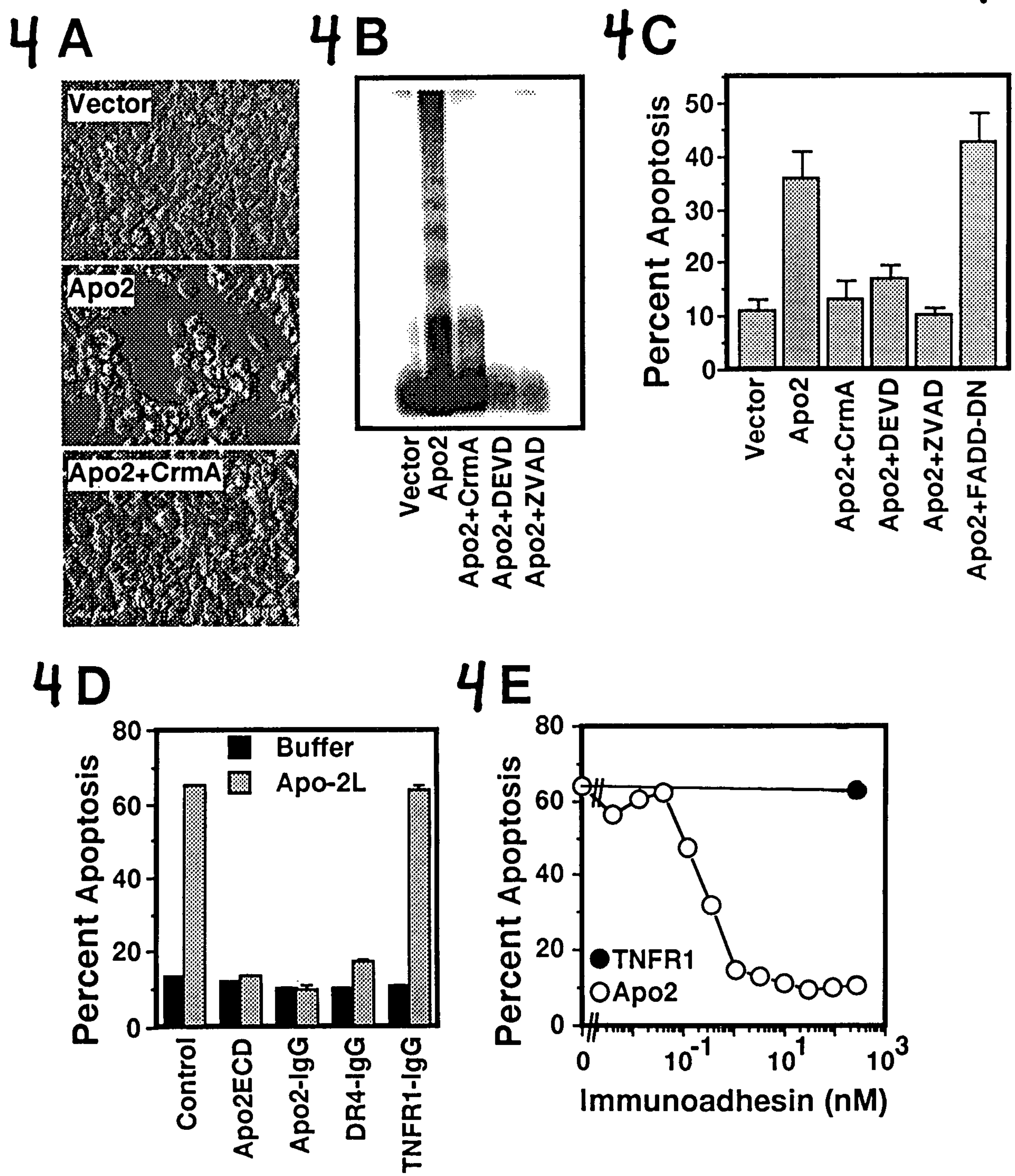
| | |
|---|---|
| Apo2 | Y T M L I K W V N K T G R D - A S V H T L L D A L E T L G E R L A K Q K I E D |
| DR4 | Y A M L M K W V N K T G R N - A S I H T L L D A L E R M E E R H A K E K I Q D |
| Apo3/DR3 | Y E M L K R W R Q Q Q P - - - A G L G A V Y A A L E R M G L D G C V E D L R S |
| TNFR1 | Y S M L A T W R R R T P R R E A T L E L L G R V L R D M D L L G C L E D I E E |
| Fas/Apo1 | - Q L L R N W H Q L H G K K E A Y - D T L I K D L K K A N L C T L A E K I Q T |

Fig. 3



IP: Anti-Flag
Nickel
Mock
ECD-Flag
ECD-Flag+His-Apo2L
Mock+His-Apo2L
Mock
ECD-Flag
ECD-Flag+His-Apo2L
Mock+His-Apo2L
28.6
18.7
His-Apo2L
WB: Anti-Apo2L
18.7
15.2
Apo2ECD-Flag
WB: Anti-Flag

Fig. 4


4A
Vector
Apo2
Apo2+CrmA
4B
Vector
Apo2
Apo2+CrmA
Apo2+DEVD
Apo2+ZVAD
4C
Percent Apoptosis
0
10
20
30
40
50
Vector
Apo2
Apo2+CrmA
Apo2+DEVD
Apo2+ZVAD
Apo2+FADD-DN
4D
Percent Apoptosis
0
20
40
60
80
Buffer
Apo-2L
Control
Apo2ECD
Apo2-IgG
DR4-IgG
TNFR1-IgG
4E
Percent Apoptosis
0
20
40
60
80
TNFR1
Apo2
0
10^-1
10^1
10^3
Immunoadhesin (nM)

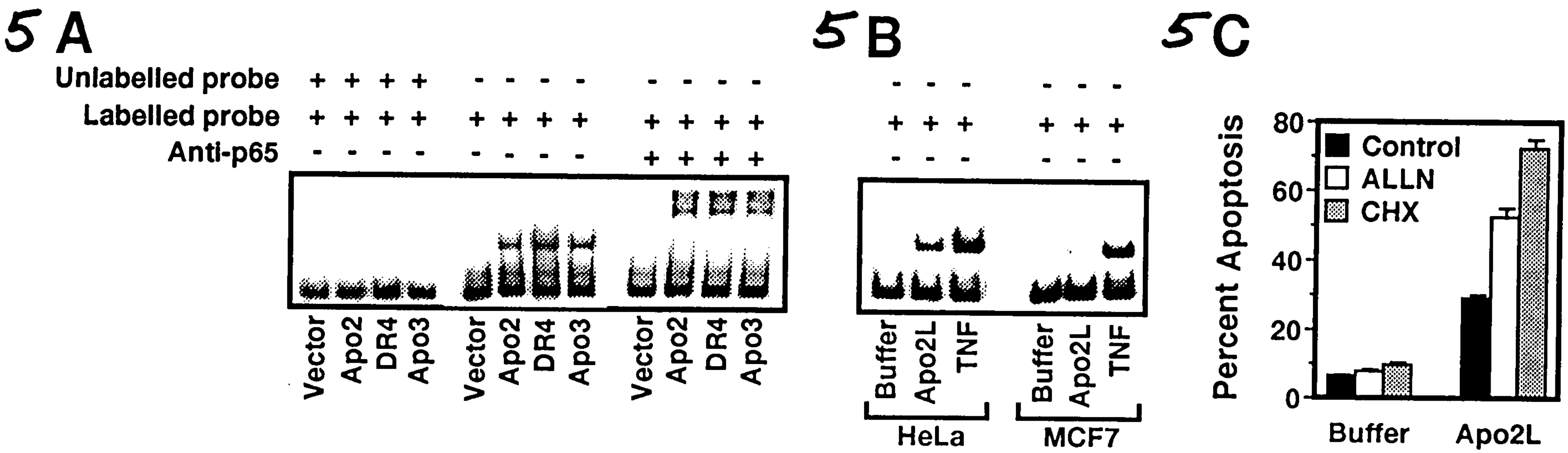

5A
Unlabelled probe
Labelled probe
Anti-p65
Vector
Apo2
DR4
Apo3
5B
Buffer
Apo2L
TNF
HeLa
MCF7
5C
Percent Apoptosis
0
20
40
60
80
Control
ALLN
CHX
Buffer
Apo2L

FIG. 5

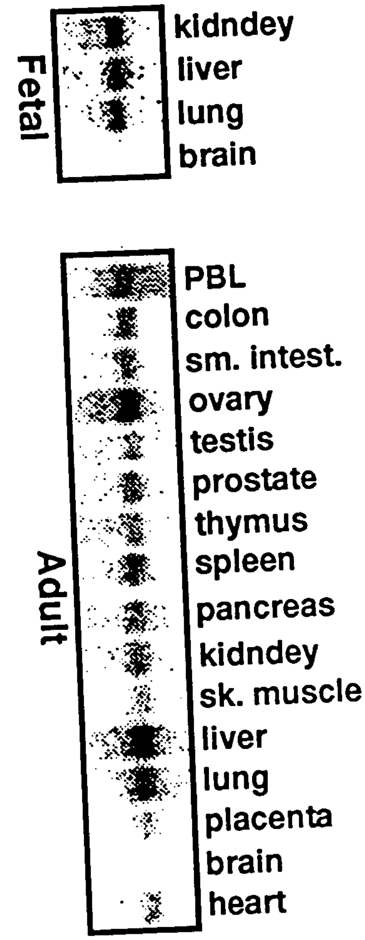

Fetal
kidndey
liver
lung
brain
Adult
PBL
colon
sm. intest.
ovary
testis
prostate
thymus
spleen
pancreas
kidndey
sk. muscle
liver
lung
placenta
brain
heart

FIG. 6

# EXHIBIT B

US006072047A

# United States Patent [19]

Rauch et al.

[11] **Patent Number:** **6,072,047**

[45] **Date of Patent:** **Jun. 6, 2000**

[54] **RECEPTOR THAT BINDS TRAIL**

[75] Inventors: **Charles Rauch**, Bainbridge Island; **Henning Walczak**, Seattle, both of Wash.

[73] Assignee: **Immunex Corporation**, Seattle, Wash.

[21] Appl. No.: **08/883,036**

[22] Filed: **Jun. 26, 1997**

**Related U.S. Application Data**

[63] Continuation-in-part of application No. 08/869,852, Jun. 4, 1997, abandoned, which is a continuation-in-part of application No. 08/829,536, Mar. 28, 1997, abandoned, which is a continuation-in-part of application No. 08/815,255, Mar. 12, 1997, abandoned, which is a continuation-in-part of application No. 08/799,861, Feb. 13, 1997, abandoned.

[51] **Int. Cl.**[7] .......................... **C07H 21/04**; C12N 15/12; C12N 15/63

[52] **U.S. Cl.** ............................ **536/23.5**; 536/24.3; 435/6; 435/320.1; 435/325; 435/252.3; 435/69.1; 530/350

[58] **Field of Search** .................................. 536/23.5, 24.3; 435/6, 320.1, 325, 252.3, 69.1; 530/350, 30

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*Assistant Examiner*—Nirmal S. Basi
*Attorney, Agent, or Firm*—Kathryn A. Anderson

[57] **ABSTRACT**

A protein designated TRAIL receptor binds the protein known as TNF-Related Apoptosis-Inducing Ligand (TRAIL). The TRAIL receptor finds use in purifying TRAIL or inhibiting activities thereof. Isolated DNA sequences encoding TRAIL-R polypeptides are provided, along with expression vectors containing the DNA sequences, and host cells transformed with such recombinant expression vectors. Antibodies that are immunoreactive with TRAIL-R are also provided.

**32 Claims, 3 Drawing Sheets**

# EXHIBIT C

08/869,852

## ABSTRACT OF THE DISCLOSURE

A protein designated TRAIL receptor binds the protein known as TNF-Related Apoptosis-Inducing Ligand (TRAIL). The TRAIL receptor finds use in purifying TRAIL or inhibiting activities thereof. Isolated DNA sequences encoding TRAIL-R polypeptides are provided, along with expression vectors containing the DNA sequences, and host cells transformed with such recombinant expression vectors. Antibodies that are immunoreactive with TRAIL-R are also provided.

Express Mail Certificate no. EM317869575US

Immunex Corporation

2625-C

# TITLE

# RECEPTOR THAT BINDS TRAIL

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application serial no. 08/829,536, filed March 28, 1997, currently pending, which is a continuation-in-part of application serial no. 08/815,255, filed March 12, 1997, currently pending, which is a continuation-in-part of application serial no. 08/799,861, filed February 13, 1997, currently pending.

## BACKGROUND OF THE INVENTION

A protein known as TNF-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family of ligands (Wiley et al., *Immunity*, 3:673-682, 1995). TRAIL has demonstrated the ability to induce apoptosis of certain transformed cells, including a number of different types of cancer cells as well as virally infected cells (PCT application WO 97/01633 and Wiley et al., *supra*).

Identification of receptor protein(s) that bind TRAIL would prove useful in further study of the biological activities of TRAIL. However, prior to the present invention, no receptor for TRAIL had been reported.

## SUMMARY OF THE INVENTION

The present invention is directed to a novel protein designated TRAIL receptor (TRAIL-R), which binds to a protein known as TNF-related apoptosis-inducing ligand (TRAIL). DNA encoding TRAIL-R, and expression vectors comprising such DNA, are provided. A method for producing TRAIL-R polypeptides comprises culturing host cells transformed with a recombinant expression vector encoding TRAIL-R, under conditions that promote expression of TRAIL-R, then recovering the expressed TRAIL-R polypeptides from the culture. Antibodies that are immunoreactive with TRAIL-R are also provided.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 presents the nucleotide sequence of a human TRAIL receptor DNA fragment, as well as the amino acid sequence encoded thereby. This DNA fragment is described in Example 3.

Figure 2 presents the results of the assay described in example 7. In the assay, a soluble TRAIL-R/Fc fusion protein blocked TRAIL-induced apoptosis of Jurkat cells.

Figure 3 presents the results of the experiment described in example 8. The indicated compounds were demonstrated to inhibit apoptosis of cells expressing TRAIL receptor.

## DETAILED DESCRIPTION OF THE INVENTION

A novel protein designated TRAIL receptor (TRAIL-R) is provided herein. TRAIL-R binds to the cytokine designated TNF-related apoptosis-inducing ligand (TRAIL). Certain uses of TRAIL-R flow from this ability to bind TRAIL, as discussed further below. TRAIL-R finds use in inhibiting biological activities of TRAIL, or in purifying TRAIL by affinity chromatography, for example.

The nucleotide sequence of the coding region of a human TRAIL receptor DNA is presented in SEQ ID NO:1. The amino acid sequence encoded by the DNA sequence of SEQ ID NO:1 is shown in SEQ ID NO:2. This sequence information identifies the TRAIL receptor protein as a member of the tumor necrosis factor receptor (TNF-R) family of receptors (reviewed in Smith et al., *Cell* 76:959-962, 1994) The extracellular domain contains cysteine rich repeats; such motifs have been reported to be important for ligand binding in other receptors of this family. TRAIL-R contains a so-called "death domain" in the cytoplasmic region; such domains in certain other receptors are associated with transduction of apoptotic signals. These and other features of the protein are discussed in more detail below.

TRAIL-R protein or immunogenic fragments thereof may be employed as immunogens to generate antibodies that are immunoreactive therewith. In one embodiment of the invention, the antibodies are monoclonal antibodies.

A human TRAIL-R protein was purified as described in example 1. In example 2, amino acid sequence information derived from fragments of TRAIL-R is presented. One embodiment of the invention is directed to a purified human TRAIL-R protein that is capable of binding TRAIL, wherein the TRAIL-R is characterized as comprising the amino acid sequence VPANEGD (amino acids 327 to 333 of SEQ ID NO:2). In another embodiment, the TRAIL-R additionally comprises the sequence ETLRQCFDDFADLVPFDSWEPLMRKLGLMDNEIKVAKAEAAGHRDTLXTML (amino acids 336 to 386 of SEQ ID NO:2, with one unknown amino acid indicated as X). Also provided are TRAIL-R fragments comprising only one of these characterizing amino acid sequences.

The nucleotide sequence of a TRAIL-R DNA fragment, and the amino acid sequence encoded thereby, are presented in Figure 1 (SEQ ID NO:3 and SEQ ID

NO:4); see example 3. The amino acid sequence presented in Figure 1 has characteristics of the so-called "death domains" found in the cytoplasmic region of certain other receptor proteins. Such domains have been reported to be associated with transduction of apoptotic signals. Cytoplasmic death domains have been identified in Fas antigen (Itoh and Nagata, *J. Biol. Chem.* 268:10932, 1993), TNF receptor type I (Tartaglia et al. *Cell* 74:845, 1993), DR3 (Chinnaiyan et al., *Science* 274:990-992, 1996), and CAR-1 (Brojatsch et al., *Cell* 87:845-855, 1996). The role of these death domains in initiating intracellular apoptotic signaling cascades is discussed further below.

SEQ ID NO:1 presents the nucleotide sequence of the coding region of a human TRAIL receptor DNA, including an initiation codon (ATG) and a termination codon (TAA). The amino acid sequence encoded by the DNA of SEQ ID NO:1 is presented in SEQ ID NO:2. The fragment depicted in Figure 1 corresponds to the region of TRAIL-R that is presented as amino acids 336 to 386 in SEQ ID NO:2.

The TRAIL-R protein of SEQ ID NO:2 includes an N-terminal hydrophobic region that functions as a signal peptide, followed by an extracellular domain, a transmembrane region comprising amino acids 211 through 231, and a C-terminal cytoplasmic domain. Computer analysis predicts that the signal peptide corresponds to residues 1 to 51 of SEQ ID NO:2. Cleavage of the signal peptide thus would yield a mature protein comprising amino acids 52 through 440 of SEQ ID NO:2. The calculated molecular weight for a mature protein containing residues 52 to 440 of SEQ ID NO:2 is about 43 kilodaltons. The next most likely computer-predicted signal peptidase cleavage sites (in descending order) occur after amino acids 50 and 58 of SEQ ID NO:2.

In another embodiment of the invention, the N-terminal residue of a mature TRAIL-R protein is the isoleucine residue at position 56 of SEQ ID NO:2. Sequences of several tryptic digest peptide fragments of TRAIL-R were determined by a combination of N-terminal sequencing and Nano-ES MS/MS (nano electrospray tandem mass spectrometry). The N-terminal amino acid of one of the peptide fragments was the isoleucine at position 56 of SEQ ID NO:2. Since this fragment was not preceded by a trypsin cleavage site, the (Ile)56 residue may correspond to the N-terminal residue resulting from cleavage of the signal peptide.

A further embodiment of the invention is directed to mature TRAIL-R having amino acid 54 as the N-terminal residue. In one preparation of TRAIL-R (a soluble TRAIL-R/Fc fusion protein expressed in CV1-EBNA cells), the signal peptide was cleaved after residue 53 of SEQ ID NO:2.

The skilled artisan will recognize that the molecular weight of particular preparations of TRAIL-R protein may differ, according to such factors as the degree of glycosylation. The glycosylation pattern of a particular preparation of TRAIL-R may vary according to the type of cells in which the protein is expressed, for example. Further, a given preparation may include multiple differentially glycosylated species of the protein. TRAIL-R polypeptides with or without associated native-pattern glycosylation are provided herein. Expression of TRAIL-R polypeptides in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules.

In one embodiment, the protein is characterized by a molecular weight within the range of about 50 to 55 kilodaltons, which is the molecular weight determined for a preparation of native, full length, human TRAIL-R. Molecular weight can be determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Example 1 presents one method for purifying a TRAIL-R protein. Jurkat cells are disrupted, and the subsequent purification process includes affinity chromatography (employing a chromatography matrix containing TRAIL), and reversed phase HPLC.

TRAIL-R polypeptides of the present invention may be purified by any suitable alternative procedure, using known protein purification techniques. In one alternative procedure, the chromatography matrix instead comprises an antibody that binds TRAIL-R. Other cell types expressing TRAIL-R (e.g., the PS-1 cells described in example 2) can be substituted for the Jurkat cells. The cells can be disrupted by any of the numerous known techniques, including freeze-thaw cycling, sonication, mechanical disruption, or by use of cell lysing agents.

The desired degree of purity depends on the intended use of the protein. A relatively high degree of purity is desired when the protein is to be administered *in vivo*, for example. Advantageously, TRAIL-R polypeptides are purified such that no protein bands corresponding to other (non-TRAIL-R) proteins are detectable upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It will be recognized by one skilled in the pertinent field that multiple bands corresponding to TRAIL-R protein may be visualized by SDS-PAGE, due to differential glycosylation, differential post-translational processing, and the like. TRAIL-R most preferably is purified to substantial homogeneity, as indicated by a single protein band upon analysis by SDS-PAGE. The protein band may be visualized by silver staining, Coomassie blue staining, or (if the protein is radiolabeled) by autoradiography.

The present invention encompasses TRAIL-R in various forms, including those that are naturally occurring or produced through various techniques such as procedures involving recombinant DNA technology. Such forms of TRAIL-R include, but are not

limited to, fragments, derivatives, variants, and oligomers of TRAIL-R, as well as fusion proteins containing TRAIL-R or fragments thereof.

TRAIL-R may be modified to create derivatives thereof by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of TRAIL-R may be prepared by linking the chemical moieties to functional groups on TRAIL-R amino acid side chains or at the N-terminus or C-terminus of a TRAIL-R polypeptide. Conjugates comprising diagnostic (detectable) or therapeutic agents attached to TRAIL-R are contemplated herein, as discussed in more detail below.

Other derivatives of TRAIL-R within the scope of this invention include covalent or aggregative conjugates of TRAIL-R polypeptides with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. Examples of fusion proteins are discussed below in connection with TRAIL-R oligomers. Further, TRAIL-R-containing fusion proteins can comprise peptides added to facilitate purification and identification of TRAIL-R. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., *Bio/Technology* 6:1204, 1988. One such peptide is the Flag® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, which is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein. A murine hybridoma designated 4E11 produces a monoclonal antibody that binds the Flag® peptide in the presence of certain divalent metal cations, as described in U.S. Patent 5,011,912, hereby incorporated by reference. The 4E11 hybridoma cell line has been deposited with the American Type Culture Collection under accession no. HB 9259. Monoclonal antibodies that bind the Flag® peptide are available from Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Connecticut.

Both cell membrane-bound and soluble (secreted) forms of TRAIL-R are provided herein. Soluble TRAIL-R may be identified (and distinguished from non-soluble membrane-bound counterparts) by separating intact cells expressing a TRAIL-R polypeptide from the culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired protein. The presence of TRAIL-R in the medium indicates that the protein was secreted from the cells and thus is a soluble form of the desired protein.

Soluble forms of receptor proteins typically lack the transmembrane region that would cause retention of the protein on the cell surface. In one embodiment of the invention, a soluble TRAIL-R polypeptide comprises the extracellular domain of the

protein. A soluble TRAIL-R polypeptide may include the cytoplasmic domain, or a portion thereof, as long as the polypeptide is secreted from the cell in which it is produced. One example of a soluble TRAIL-R is a soluble human TRAIL-R comprising amino acids 52 to 210 of SEQ ID NO:2. Other soluble TRAIL-R polypeptides include, but are not limited to, polypeptides comprising amino acids x to 210 of SEQ ID NO:2, wherein x is an integer from 51 through 59.

Soluble forms of TRAIL-R possess certain advantages over the membrane-bound form of the protein. Purification of the protein from recombinant host cells is facilitated, since the soluble proteins are secreted from the cells. Further, soluble proteins are generally more suitable for certain applications, e.g., for intravenous administration.

TRAIL-R fragments are provided herein. Such fragments may be prepared by any of a number of conventional techniques. Desired peptide fragments may be chemically synthesized. An alternative involves generating TRAIL-R fragments by enzymatic digestion, e.g., by treating the protein with an enzyme known to cleave proteins at sites defined by particular amino acid residues. Yet another suitable technique involves isolating and amplifying a DNA fragment encoding a desired polypeptide fragment, by polymerase chain reaction (PCR). Oligonucleotides that define the desired termini of the DNA fragment are employed as the 5' and 3' primers in the PCR.

Examples of fragments are those comprising at least 20, or at least 30, contiguous amino acids of the sequence of SEQ ID NO:2. Fragments derived from the cytoplasmic domain find use in studies of TRAIL-R-mediated signal transduction, and in regulating cellular processes associated with transduction of biological signals. TRAIL-R polypeptide fragments also may be employed as immunogens, in generating antibodies. Particular embodiments are directed to TRAIL-R polypeptide fragments that retain the ability to bind TRAIL. Such a fragment may be a soluble TRAIL-R polypeptide, as described above.

Naturally occurring variants of the TRAIL-R protein of SEQ ID NO:2 are provided herein. Such variants include, for example, proteins that result from alternate mRNA splicing events or from proteolytic cleavage of the TRAIL-R protein. Alternate splicing of mRNA may, for example, yield a truncated but biologically active TRAIL-R protein, such as a naturally occurring soluble form of the protein. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the TRAIL-R protein (generally from 1-5 terminal amino

acids). TRAIL-R proteins in which differences in amino acid sequence are attributable to genetic polymorphism (allelic variation among individuals producing the protein) are also contemplated herein.

The skilled artisan will also recognize that the position(s) at which the signal peptide is cleaved may differ from that predicted by computer program, and may vary according to such factors as the type of host cells employed in expressing a recombinant TRAIL-R polypeptide. A protein preparation may include a mixture of protein molecules having different N-terminal amino acids, resulting from cleavage of the signal peptide at more than one site. As discussed above, particular embodiments of mature TRAIL-R proteins provided herein include, but are not limited to, proteins having the residue at position 51, 52, 54, 56, or 59 of SEQ ID NO:2 as the N-terminal amino acid.

Regarding the discussion herein of various domains of TRAIL-R protein, the skilled artisan will recognize that the above-described boundaries of such regions of the protein are approximate. To illustrate, the boundaries of the transmembrane region (which may be predicted by using computer programs available for that purpose) may differ from those described above. Thus, soluble TRAIL-R polypeptides in which the C-terminus of the extracellular domain differs from the residue so identified above are contemplated herein.

Other naturally occurring TRAIL-R DNAs and polypeptides include those derived from non-human species. Homologs of the human TRAIL-R of SEQ ID NO:2, from other mammalian species, are contemplated herein, for example. Probes based on the human DNA sequence of SEQ ID NO:3 or SEQ ID NO:1 may be used to screen cDNA libraries derived from other mammalian species, using conventional cross-species hybridization techniques.

TRAIL-R DNA sequences may vary from the native sequences disclosed herein. Due to the known degeneracy of the genetic code, wherein more than one codon can encode the same amino acid, a DNA sequence can vary from that shown in SEQ ID NO:1 and still encode a TRAIL-R protein having the amino acid sequence of SEQ ID NO:2. Such variant DNA sequences may result from silent mutations (e.g., occurring during PCR amplification), or may be the product of deliberate mutagenesis of a native sequence. Thus, among the DNA sequences provided herein are native TRAIL-R sequences (e.g., cDNA comprising the nucleotide sequence presented in SEQ ID NO:1) and DNA that is degenerate as a result of the genetic code to a native TRAIL-R DNA sequence.

Among the TRAIL-R polypeptides provided herein are variants of native TRAIL-R polypeptides that retain a biological activity of a native TRAIL-R. Such variants include polypeptides that are substantially homologous to native TRAIL-R, but which have an amino acid sequence different from that of a native TRAIL-R because of one or more deletions, insertions or substitutions. Particular embodiments include, but are not limited to, TRAIL-R polypeptides that comprise from one to ten deletions, insertions or substitutions of amino acid residues, when compared to a native TRAIL-R sequence. The TRAIL-R-encoding DNAs of the present invention include variants that differ from a native TRAIL-R DNA sequence because of one or more deletions, insertions or substitutions, but that encode a biologically active TRAIL-R polypeptide. One biological activity of TRAIL-R is the ability to bind TRAIL.

Nucleic acid molecules capable of hybridizing to the DNA of SEQ ID NO:1 or SEQ ID NO:3 under moderately stringent or highly stringent conditions, and which encode a biologically active TRAIL-R, are provided herein. Such hybridizing nucleic acids include, but are not limited to, variant DNA sequences and DNA derived from non-human species, e.g., non-human mammals.

Moderately stringent conditions include conditions described in, for example, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Vol. 1, pp 1.101-104, Cold Spring Harbor Laboratory Press, 1989. Conditions of moderate stringency, as defined by Sambrook et al., include use of a prewashing solution of 5X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization conditions of about 55°C, 5 X SSC, overnight. Highly stringent conditions include higher temperatures of hybridization and washing. One embodiment of the invention is directed to DNA sequences that will hybridize to the DNA of SEQ ID NOS:1 or 3 under highly stringent conditions, wherein said conditions include hybridization at 68°C followed by washing in 0.1X SSC/0.1% SDS at 63-68°C.

Certain DNAs and polypeptides provided herein comprise nucleotide or amino acid sequences, respectively, that are at least 80% identical to a native TRAIL-R sequence. Also contemplated are embodiments in which a TRAIL-R DNA or polypeptide comprises a sequence that is at least 90% identical, at least 95% identical, or at least 98% identical to a native TRAIL-R sequence. The percent identity may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0 described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides,

and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res. 14*:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

In particular embodiments of the invention, a variant TRAIL-R polypeptide differs in amino acid sequence from a native TRAIL-R, but is substantially equivalent to a native TRAIL-R in a biological activity. One example is a variant TRAIL-R that binds TRAIL with essentially the same binding affinity as does a native TRAIL-R. Binding affinity can be measured by conventional procedures, e.g., as described in U.S. Patent no. 5,512,457.

Variant amino acid sequences may comprise conservative substitution(s), meaning that one or more amino acid residues of a native TRAIL-R is replaced by a different residue, but that the conservatively substituted TRAIL-R polypeptide retains a desired biological activity of the native protein (e.g., the ability to bind TRAIL). A given amino acid may be replaced by a residue having similar physiochemical characteristics. Examples of conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other conservative substitutions, e.g., involving substitutions of entire regions having similar hydrophobicity characteristics, are well known.

In another example of variants, sequences encoding Cys residues that are not essential for biological activity can be altered to cause the Cys residues to be deleted or replaced with other amino acids, preventing formation of incorrect intramolecular disulfide bridges upon renaturation. Certain receptors of the TNF-R family contain cysteine-rich repeat motifs in their extracellular domains (Marsters et al., J. Biol. Chem. 267:5747-5750, 1992). These repeats are believed to be important for ligand binding. To illustrate, Marsters et al., *supra*, reported that soluble TNF-R type 1 polypeptides lacking one of the repeats exhibited a ten fold reduction in binding affinity for TNF$\alpha$ and TNFß; deletion of the second repeat resulted in a complete loss of detectable binding of the ligands. The human TRAIL-R of SEQ ID NO:2 contains two such cysteine rich repeats, the first including residues 94 through 137, and the second including residues 138 through 178. Cysteine residues within these cysteine rich domains advantageously remain unaltered in TRAIL-R variants, when retention of TRAIL-binding activity is desired.

Other variants are prepared by modification of adjacent dibasic amino acid residues, to enhance expression in yeast systems in which KEX2 protease activity is present. EP 212,914 discloses the use of site-specific mutagenesis to inactivate KEX2 protease processing sites in a protein. KEX2 protease processing sites are inactivated by deleting, adding or substituting residues to alter Arg-Arg, Arg-Lys, and Lys-Arg pairs to eliminate the occurrence of these adjacent basic residues. Mature human TRAIL-R contains such adjacent basic residue pairs at amino acids 72-73, 154-155, 322-323, 323-324, and 359-360 of SEQ ID NO:2. Lys-Lys pairings are considerably less susceptible to KEX2 cleavage, and conversion of Arg-Lys or Lys-Arg to Lys-Lys represents a conservative and preferred approach to inactivating KEX2 sites.

TRAIL-R polypeptides, including variants and fragments thereof, can be tested for biological activity in any suitable assay. The ability of a TRAIL-R polypeptide to bind TRAIL can be confirmed in conventional binding assays, examples of which are described below.

Expression Systems

The present invention also provides recombinant cloning and expression vectors containing TRAIL-R DNA, as well as host cell containing the recombinant vectors. Expression vectors comprising TRAIL-R DNA may be used to prepare TRAIL-R polypeptides encoded by the DNA. A method for producing TRAIL-R polypeptides comprises culturing host cells transformed with a recombinant expression vector encoding TRAIL-R, under conditions that promote expression of TRAIL-R, then recovering the expressed TRAIL-R polypeptides from the culture. The skilled artisan will recognize that the procedure for purifying the expressed TRAIL-R will vary according to such factors as the type of host cells employed, and whether the TRAIL-R is membrane-bound or a soluble form that is secreted from the host cell.

Any suitable expression system may be employed. The vectors include a DNA encoding a TRAIL-R polypeptide, operably linked to suitable transcriptional or translational regulatory nucleotide sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the TRAIL-R DNA sequence. Thus, a promoter nucleotide sequence is operably linked to an TRAIL-R DNA sequence if the promoter nucleotide sequence controls the transcription of the TRAIL-R DNA sequence. An origin of

replication that confers the ability to replicate in the desired host cells, and a selection gene by which transformants are identified, are generally incorporated into the expression vector.

In addition, a sequence encoding an appropriate signal peptide (native or heterologous) can be incorporated into expression vectors. A DNA sequence for a signal peptide (secretory leader) may be fused in frame to the TRAIL-R sequence so that the TRAIL-R is initially translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells promotes extracellular secretion of the TRAIL-R polypeptide. The signal peptide is cleaved from the TRAIL-R polypeptide upon secretion of TRAIL-R from the cell.

Suitable host cells for expression of TRAIL-R polypeptides include prokaryotes, yeast or higher eukaryotic cells. Mammalian or insect cells are generally preferred for use as host cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual,* Elsevier, New York, (1985). Cell-free translation systems could also be employed to produce TRAIL-R polypeptides using RNAs derived from DNA constructs disclosed herein.

Prokaryotes include gram negative or gram positive organisms, for example, *E. coli* or *Bacilli*. Suitable prokaryotic host cells for transformation include, for example, *E. coli, Bacillus subtilis, Salmonella typhimurium,* and various other species within the genera *Pseudomonas, Streptomyces,* and *Staphylococcus*. In a prokaryotic host cell, such as *E. coli*, a TRAIL-R polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant TRAIL-R polypeptide.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those derived from commercially available plasmids such as the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. An appropriate promoter and a TRAIL-R DNA sequence are inserted into the pBR322 vector. Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA).

Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include β-lactamase (penicillinase), lactose promoter system (Chang et al., *Nature 275*:615, 1978; and Goeddel et al., *Nature 281*:544, 1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res. 8*:4057, 1980; and EP-A-36776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell expression system employs a phage λ $P_L$ promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ $P_L$ promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9, ATCC 37092) and pPLc28 (resident in *E. coli* RR1, ATCC 53082).

TRAIL-R alternatively may be expressed in yeast host cells, preferably from the *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* or *Kluyveromyces*, may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem. 255*:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg. 7*:149, 1968; and Holland et al., *Biochem. 17*:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EPA-73,657. Another alternative is the glucose-repressible ADH2 promoter described by Russell et al. (*J. Biol. Chem. 258*:2674, 1982) and Beier et al. (*Nature 300*:724, 1982). Shuttle vectors replicable in both yeast and *E. coli* may be constructed by inserting DNA sequences from pBR322 for selection and replication in *E. coli* ($Amp^r$ gene and origin of replication) into the above-described yeast vectors.

The yeast α-factor leader sequence may be employed to direct secretion of the TRAIL polypeptide. The α-factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. *See,* e.g., Kurjan et al., *Cell 30*:933, 1982 and Bitter et al., *Proc. Natl. Acad. Sci. USA 81*:5330, 1984. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified

near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA 75*:1929, 1978. The Hinnen et al. protocol selects for $Trp^+$ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 µg/ml adenine and 20 µg/ml uracil.

Yeast host cells transformed by vectors containing an ADH2 promoter sequence may be grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 µg/ml adenine and 80 µg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems also may be employed to express recombinant TRAIL-R polypeptides. Bacculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988). Established cell lines of mammalian origin also may be employed. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman et al., *Cell 23*:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines, and the CV1/EBNA cell line derived from the African green monkey kidney cell line CV1 (ATCC CCL 70) as described by McMahan et al. (*EMBO J*. 10: 2821, 1991).

Transcriptional and translational control sequences for mammalian host cell expression vectors may be excised from viral genomes. Commonly used promoter sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication (Fiers et al., *Nature 273*:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the SV40 viral origin of replication site is included.

Expression vectors for use in mammalian host cells can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol. 3*:280, 1983), for example. A useful system for stable high level expression of mammalian cDNAs in C127 murine

mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol.* 23:935, 1986). A high expression vector, PMLSV N1/N4, described by Cosman et al., *Nature 312*:768, 1984 has been deposited as ATCC 39890. Additional mammalian expression vectors are described in EP-A-0367566, and in WO 91/18982. As one alternative, the vector may be derived from a retrovirus.

Overexpression of full length TRAIL-R has resulted in membrane blebbing and nuclear condensation of transfected CV-1/EBNA cells, indicating that the mechanism of cell death was apoptosis. For host cells in which such TRAIL-R-mediated apoptosis occurs, a suitable apoptosis inhibitor may be included in the expression system.

To inhibit TRAIL-R-induced apoptosis of host cells expressing recombinant TRAIL-R, the cells may be co-transfected with an expression vector encoding a polypeptide that functions as an apoptosis inhibitor. Expression vectors encoding such polypeptides can be prepared by conventional procedures. Another approach involves adding an apoptosis inhibitor to the culture medium. The use of poxvirus CrmA, baculovirus P35, a C-terminal fragment of FADD, and the tripeptide derivative zVAD-fmk, to reduce death of host cells is illustrated in examples 6 and 8.

zVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) is a tripeptide based compound, available from Enzyme System Products, Dublin, California. As illustrated in example 8, zVAD-fmk may be added to the medium in which cells expressing TRAIL-R are cultured.

The 38-kilodalton cowpox-derived protein that was subsequently designated CrmA is described in Pickup et al. (*Proc. Natl. Acad. Sci. USA* 83:7698-7702, 1986; hereby incorporated by reference). Sequence information for CrmA is presented in Figure 4 of Pickup et al., *supra.* One approach to producing and purifying CrmA protein is described in Ray et al. (*Cell*, 69:597-604, 1992; hereby incorporated by reference).

A 35-kilodalton protein encoded by *Autographa californica* nuclear polyhedrosis virus, a baculovirus, is described in Friesen and Miller (*J. Virol.* 61:2264-2272, 1987; hereby incorporated by reference). Sequence information for this protein, designated baculovirus p35 herein, is presented in Figure 5 of Friesen and Miller, *supra.*

The death domain-containing cytoplasmic protein FADD (also known as MORT1) is described in Boldin et al. (*J. Biol. Chem.* 270:7795-7798, 1995; hereby incorporated by reference). FADD has been reported to associate, directly or indirectly, with the cytoplasmic death domain of certain receptors that mediate apoptosis (Boldin et al., *Cell* 85:803-815, June 1996; Hsu et al., *Cell* 84:299-308, January 1996).

In one embodiment of the present invention, truncated FADD polypeptides that include the death domain (located in the C-terminal portion of the protein), but lack the N-terminal region to which apoptosis effector functions have been attributed, are employed to reduce apoptosis. The use of certain FADD deletion mutant polypeptides, truncated at the N-terminus, to inhibit death of cells expressing other apoptosis-inducing receptors, is described in Hsu et al. (*Cell* 84:299-308, 1996; hereby incorporated by reference).

This approach is illustrated in example 8, which employs one suitable FADD-dominant negative (FADD-DN) polypeptide, having an amino acid sequence corresponding to amino acids 117 through 245 of the MORT1 amino acid sequence presented in Boldin et al. (*J. Biol. Chem.* 270:7795-7798, 1995). In example 8, cells were co-transfected with a TRAIL-R-encoding expression vector, and with an expression vector encoding the above-described Flag® peptide, fused to the N-terminus of the FADD-DN polypeptide.

While not wishing to be bound by theory, one possible explanation is that the C-terminal fragments of FADD associate with the intracellular death domain of the receptor, but lack the N-terminal portion of the protein that is necessary for effecting apoptosis (Hsu et al., *Cell* 84:299-308, January 1996; Boldin et al., *Cell* 85:803-815, June 1996). The truncated FADD thereby may block association of endogenous, full length FADD with the receptor's death domain; consequently, the apoptosis that would be initiated by such endogenous FADD is inhibited.

Other apoptosis inhibitors useful in expression systems of the present invention can be identified in conventional assay procedures. One such assay, in which compounds are tested for the ability to reduce apoptosis of cells expressing TRAIL-R, is described in example 8.

Poxvirus CrmA, baculovirus P35, and zVAD-fmk are viral caspase inhibitors. Other caspase inhibitors may be tested for the ability to reduce TRAIL-R-mediated cell death.

The use of CrmA, baculovirus p35, and certain peptide derivatives (including zVAD-fmk) as inhibitors of apoptosis in particular cells/systems is discussed in Sarin et al. (*J. Exp. Med.* 184:2445-2450, Dec. 1996; hereby incorporated by reference). The role of interleukin-1ß converting enzyme (ICE) family proteases in signal transduction cascades leading to programmed cell death, and the use of inhibitors of such proteases to block apoptosis, is discussed in Sarin et al., *supra*, and Muzio et al., *Cell* 85:817-827, 1996).

Apoptosis inhibitors generally need not be employed for expression of TRAIL-R polypeptides lacking the cytoplasmic domain (i.e., lacking the region of the protein involved in signal transduction). Thus, expression systems for producing soluble TRAIL-R polypeptides comprising only the extracellular domain (or a fragment thereof) need not include one of the above-described apoptosis inhibitors.

Regarding signal peptides that may be employed in producing TRAIL-R, the native signal peptide of TRAIL-R may be replaced by a heterologous signal peptide or leader sequence, if desired. The choice of signal peptide or leader may depend on factors such as the type of host cells in which the recombinant TRAIL-R is to be produced. To illustrate, examples of heterologous signal peptides that are functional in mammalian host cells include the signal sequence for interleukin-7 (IL-7) described in United States Patent 4,965,195, the signal sequence for interleukin-2 receptor described in Cosman et al., *Nature* 312:768 (1984); the interleukin-4 receptor signal peptide described in EP 367,566; the type I interleukin-1 receptor signal peptide described in U.S. Patent 4,968,607; and the type II interleukin-1 receptor signal peptide described in EP 460,846.

Oligomeric Forms of TRAIL-R

Encompassed by the present invention are oligomers that contain TRAIL-R polypeptides. TRAIL-R oligomers may be in the form of covalently-linked or non-covalently-linked dimers, trimers, or higher oligomers.

One embodiment of the invention is directed to oligomers comprising multiple TRAIL-R polypeptides joined *via* covalent or non-covalent interactions between peptide moieties fused to the TRAIL-R polypeptides. Such peptides may be peptide linkers (spacers), or peptides that have the property of promoting oligomerization. Leucine zippers and certain polypeptides derived from antibodies are among the peptides that can promote oligomerization of TRAIL-R polypeptides attached thereto, as described in more detail below.

In particular embodiments, the oligomers comprise from two to four TRAIL-R polypeptides. The TRAIL-R moieties of the oligomer may be soluble polypeptides, as described above.

As one alternative, a TRAIL-R oligomer is prepared using polypeptides derived from immunoglobulins. Preparation of fusion proteins comprising certain heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi et al. (*PNAS USA* 88:10535, 1991); Byrn et al. (*Nature* 344:677, 1990); and Hollenbaugh and Aruffo

("Construction of Immunoglobulin Fusion Proteins", in *Current Protocols in Immunology*, Suppl. 4, pages 10.19.1 - 10.19.11, 1992).

One embodiment of the present invention is directed to a TRAIL-R dimer comprising two fusion proteins created by fusing TRAIL-R to the Fc region of an antibody. A gene fusion encoding the TRAIL-R/Fc fusion protein is inserted into an appropriate expression vector. TRAIL-R/Fc fusion proteins are expressed in host cells transformed with the recombinant expression vector, and allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between the Fc moieties to yield divalent TRAIL-R.

Provided herein are fusion proteins comprising a TRAIL-R polypeptide fused to an Fc polypeptide derived from an antibody. DNA encoding such fusion proteins, as well as dimers containing two fusion proteins joined *via* disulfide bonds between the Fc moieties thereof, are also provided. The term "Fc polypeptide" as used herein includes native and mutein forms of polypeptides derived from the Fc region of an antibody. Truncated forms of such polypeptides containing the hinge region that promotes dimerization are also included.

One suitable Fc polypeptide, described in PCT application WO 93/10151 (hereby incorporated by reference), is a single chain polypeptide extending from the N-terminal hinge region to the native C-terminus of the Fc region of a human IgG1 antibody. Another useful Fc polypeptide is the Fc mutein described in U.S. Patent 5,457,035 and in Baum et al., (*EMBO J.* 13:3992-4001, 1994). The amino acid sequence of this mutein is identical to that of the native Fc sequence presented in WO 93/10151, except that amino acid 19 has been changed from Leu to Ala, amino acid 20 has been changed from Leu to Glu, and amino acid 22 has been changed from Gly to Ala. The mutein exhibits reduced affinity for Fc receptors.

In other embodiments, TRAIL-R may be substituted for the variable portion of an antibody heavy or light chain. If fusion proteins are made with both heavy and light chains of an antibody, it is possible to form a TRAIL-R oligomer with as many as four TRAIL-R extracellular regions.

Alternatively, the oligomer is a fusion protein comprising multiple TRAIL-R polypeptides, with or without peptide linkers (spacer peptides). Among the suitable peptide linkers are those described in U.S. Patents 4,751,180 and 4,935,233, which are hereby incorporated by reference. A DNA sequence encoding a desired peptide linker may be inserted between, and in the same reading frame as, the DNA sequences encoding TRAIL-R, using any suitable conventional technique. For example, a chemically synthesized oligonucleotide encoding the linker may be ligated between

sequences encoding TRAIL-R. In particular embodiments, a fusion protein comprises from two to four soluble TRAIL-R polypeptides, separated by peptide linkers.

Another method for preparing oligomeric TRAIL-R involves use of a leucine zipper. Leucine zipper domains are peptides that promote oligomerization of the proteins in which they are found. Leucine zippers were originally identified in several DNA-binding proteins (Landschulz et al., *Science* 240:1759, 1988), and have since been found in a variety of different proteins. Among the known leucine zippers are naturally occurring peptides and derivatives thereof that dimerize or trimerize.

Examples of leucine zipper domains suitable for producing soluble oligomeric proteins are described in PCT application WO 94/10308, and the leucine zipper derived from lung surfactant protein D (SPD) described in Hoppe et al. (*FEBS Letters* 344:191, 1994), hereby incorporated by reference. The use of a modified leucine zipper that allows for stable trimerization of a heterologous protein fused thereto is described in Fanslow et al. (*Semin. Immunol.* 6:267-278, 1994). Recombinant fusion proteins comprising a soluble TRAIL-R polypeptide fused to a leucine zipper peptide are expressed in suitable host cells, and the soluble oligomeric TRAIL-R that forms is recovered from the culture supernatant.

Oligomeric TRAIL-R has the property of bivalent, trivalent, etc. binding sites for TRAIL. The above-described fusion proteins comprising Fc moieties (and oligomers formed therefrom) offer the advantage of facile purification by affinity chromatography over Protein A or Protein G columns. DNA sequences encoding oligomeric TRAIL-R, or encoding fusion proteins useful in preparing TRAIL-R oligomers, are provided herein.

Assays

TRAIL-R proteins (including fragments, variants, oligomers, and other forms of TRAIL-R) may be tested for the ability to bind TRAIL in any suitable assay, such as a conventional binding assay. To illustrate, TRAIL-R may be labeled with a detectable reagent (e.g., a radionuclide, chromophore, enzyme that catalyzes a colorimetric or fluorometric reaction, and the like). The labeled TRAIL-R is contacted with cells expressing TRAIL. The cells then are washed to remove unbound labeled TRAIL-R, and the presence of cell-bound label is determined by a suitable technique, chosen according to the nature of the label.

One example of a binding assay procedure is as follows. A recombinant expression vector containing TRAIL cDNA is constructed, e.g., as described in in PCT application WO 97/01633, hereby incorporated by reference. DNA and amino acid

sequence information for human and mouse TRAIL is presented in WO 97/01633. TRAIL comprises an N-terminal cytoplasmic domain, a transmembrane region, and a C-terminal extracellular domain. CV1-EBNA-1 cells in 10 $cm^2$ dishes are transfected with the recombinant expression vector. CV-1/EBNA-1 cells (ATCC CRL 10478) constitutively express EBV nuclear antigen-1 driven from the CMV immediate-early enhancer/promoter. CV1-EBNA-1 was derived from the African Green Monkey kidney cell line CV-1 (ATCC CCL 70), as described by McMahan et al. (*EMBO J.* 10:2821, 1991).

The transfected cells are cultured for 24 hours, and the cells in each dish then are split into a 24-well plate. After culturing an additional 48 hours, the transfected cells (about 4 x $10^4$ cells/well) are washed with BM-NFDM, which is binding medium (RPMI 1640 containing 25 mg/ml bovine serum albumin, 2 mg/ml sodium azide, 20 mM Hepes pH 7.2) to which 50 mg/ml nonfat dry milk has been added. The cells then are incubated for 1 hour at 37°C with various concentrations of a soluble TRAIL-R/Fc fusion protein. Cells then are washed and incubated with a constant saturating concentration of a $^{125}$I-mouse anti-human IgG in binding medium, with gentle agitation for 1 hour at 37°C. After extensive washing, cells are released *via* trypsinization.

The mouse anti-human IgG employed above is directed against the Fc region of human IgG and can be obtained from Jackson Immunoresearch Laboratories, Inc., West Grove, PA. The antibody is radioiodinated using the standard chloramine-T method. The antibody will bind to the Fc portion of any TRAIL-R/Fc protein that has bound to the cells. In all assays, non-specific binding of $^{125}$I-antibody is assayed in the absence of TRAIL-R/Fc, as well as in the presence of TRAIL-R/Fc and a 200-fold molar excess of unlabeled mouse anti-human IgG antibody.

Cell-bound $^{125}$I-antibody is quantified on a Packard Autogamma counter. Affinity calculations (Scatchard, *Ann. N.Y. Acad. Sci.* 51:660, 1949) are generated on RS/1 (BBN Software, Boston, MA) run on a Microvax computer.

Another type of suitable binding assay is a competitive binding assay. To illustrate, biological activity of a TRAIL-R variant may be determined by assaying for the variant's ability to compete with a native TRAIL-R for binding to TRAIL.

Competitive binding assays can be performed by conventional methodology. Reagents that may be employed in competitive binding assays include radiolabeled TRAIL-R and intact cells expressing TRAIL (endogenous or recombinant) on the cell surface. For example, a radiolabeled soluble TRAIL-R fragment can be used to compete with a soluble TRAIL-R variant for binding to cell surface TRAIL. Instead of intact cells, one could substitute a soluble TRAIL/Fc fusion protein bound to a solid

phase through the interaction of Protein A or Protein G (on the solid phase) with the Fc moiety. Chromatography columns that contain Protein A and Protein G include those available from Pharmacia Biotech, Inc., Piscataway, NJ. Another type of competitive binding assay utilizes radiolabeled soluble TRAIL, such as a soluble TRAIL/Fc fusion protein, and intact cells expressing TRAIL-R. Qualitative results can be obtained by competitive autoradiographic plate binding assays, while Scatchard plots (Scatchard, *Ann. N.Y. Acad. Sci.* 51:660, 1949) may be utilized to generate quantitative results.

Another type of assay for biological activity involves testing a TRAIL-R polypeptide for the ability to block TRAIL-mediated apoptosis of target cells, such as the human leukemic T-cell line known as Jurkat cells, for example. TRAIL-mediated apoptosis of the cell line designated Jurkat clone E6-1 (ATCC TIB 152) is demonstrated in assay procedures described in PCT application WO 97/01633, hereby incorporated by reference.

Uses of TRAIL-R

Uses of TRAIL-R include, but are not limited to, the following. Certain of these uses of TRAIL-R flow from its ability to bind TRAIL.

TRAIL-R finds use as a protein purification reagent. TRAIL-R polypeptides may be attached to a solid support material and used to purify TRAIL proteins by affinity chromatography. In particular embodiments, a TRAIL-R polypeptide (in any form described herein that is capable of binding TRAIL) is attached to a solid support by conventional procedures. As one example, chromatography columns containing functional groups that will react with functional groups on amino acid side chains of proteins are available (Pharmacia Biotech, Inc., Piscataway, NJ). In an alternative, a TRAIL-R/Fc protein is attached to Protein A- or Protein G-containing chromatography columns through interaction with the Fc moiety.

TRAIL-R proteins also find use in measuring the biological activity of TRAIL proteins in terms of their binding affinity for TRAIL-R. TRAIL-R proteins thus may be employed by those conducting "quality assurance" studies, e.g., to monitor shelf life and stability of TRAIL protein under different conditions. To illustrate, TRAIL-R may be employed in a binding affinity study to measure the biological activity of a TRAIL protein that has been stored at different temperatures, or produced in different cell types. TRAIL-R also may be used to determine whether biological activity is retained after modification of a TRAIL protein (e.g., chemical modification, truncation, mutation, etc.). The binding affinity of the modified TRAIL protein for TRAIL-R is compared to that of an unmodified TRAIL protein to detect any adverse impact of the

modifications on biological activity of TRAIL. The biological activity of a TRAIL protein thus can be ascertained before it is used in a research study, for example.

TRAIL-R also finds use in purifying or identifying cells that express TRAIL on the cell surface. TRAIL-R polypeptides are bound to a solid phase such as a column chromatography matrix or a similar suitable substrate. For example, magnetic microspheres can be coated with TRAIL-R and held in an incubation vessel through a magnetic field. Suspensions of cell mixtures containing TRAIL-expressing cells are contacted with the solid phase having TRAIL-R thereon. Cells expressing TRAIL on the cell surface bind to the fixed TRAIL-R, and unbound cells then are washed away.

Alternatively, TRAIL-R can be conjugated to a detectable moiety, then incubated with cells to be tested for TRAIL expression. After incubation, unbound labeled TRAIL-R is removed and the presence or absence of the detectable moiety on the cells is determined.

In a further alternative, mixtures of cells suspected of containing TRAIL$^+$ cells are incubated with biotinylated TRAIL-R. Incubation periods are typically at least one hour in duration to ensure sufficient binding. The resulting mixture then is passed through a column packed with avidin-coated beads, whereby the high affinity of biotin for avidin provides binding of the desired cells to the beads. Procedures for using avidin-coated beads are known (see Berenson, et al. *J. Cell. Biochem.*, 10D:239, 1986). Washing to remove unbound material, and the release of the bound cells, are performed using conventional methods.

TRAIL-R polypeptides also find use as carriers for delivering agents attached thereto to cells bearing TRAIL. Cells expressing TRAIL include those identified in Wiley et al. (*Immunity*, 3:673-682, 1995). TRAIL-R proteins thus can be used to deliver diagnostic or therapeutic agents to such cells (or to other cell types found to express TRAIL on the cell surface) in *in vitro* or *in vivo* procedures.

Detectable (diagnostic) and therapeutic agents that may be attached to a TRAIL-R polypeptide include, but are not limited to, toxins, other cytotoxic agents, drugs, radionuclides, chromophores, enzymes that catalyze a colorimetric or fluorometric reaction, and the like, with the particular agent being chosen according to the intended application. Among the toxins are ricin, abrin, diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A, ribosomal inactivating proteins, mycotoxins such as trichothecenes, and derivatives and fragments (e.g., single chains) thereof. Radionuclides suitable for diagnostic use include, but are not limited to, $^{123}I$, $^{131}I$, $^{99m}Tc$, $^{111}In$, and $^{76}Br$. Examples of radionuclides suitable for therapeutic use are $^{131}I$, $^{211}At$, $^{77}Br$, $^{186}Re$, $^{188}Re$, $^{212}Pb$, $^{212}Bi$, $^{109}Pd$, $^{64}Cu$, and $^{67}Cu$.

Such agents may be attached to the TRAIL-R by any suitable conventional procedure. TRAIL-R, being a protein, comprises functional groups on amino acid side chains that can be reacted with functional groups on a desired agent to form covalent bonds, for example. Alternatively, the protein or agent may be derivatized to generate or attach a desired reactive functional group. The derivatization may involve attachment of one of the bifunctional coupling reagents available for attaching various molecules to proteins (Pierce Chemical Company, Rockford, Illinois). A number of techniques for radiolabeling proteins are known. Radionuclide metals may be attached to TRAIL-R by using a suitable bifunctional chelating agent, for example.

Conjugates comprising TRAIL-R and a suitable diagnostic or therapeutic agent (preferably covalently linked) are thus prepared. The conjugates are administered or otherwise employed in an amount appropriate for the particular application.

TRAIL-R DNA and polypeptides of the present invention may be used in developing treatments for any disorder mediated (directly or indirectly) by defective, or insufficient amounts of, TRAIL-R. TRAIL-R polypeptides may be administered to a mammal afflicted with such a disorder. Alternatively, a gene therapy approach may be taken. Disclosure herein of native TRAIL-R nucleotide sequences permits the detection of defective TRAIL-R genes, and the replacement thereof with normal TRAIL-R-encoding genes. Defective genes may be detected in *in vitro* diagnostic assays, and by comparision of a native TRAIL-R nucleotide sequence disclosed herein with that of a TRAIL-R gene derived from a person suspected of harboring a defect in this gene.

Another use of the protein of the present invention is as a research tool for studying the biological effects that result from inhibiting TRAIL/TRAIL-R interactions on different cell types. TRAIL-R polypeptides also may be employed in *in vitro* assays for detecting TRAIL or TRAIL-R or the interactions thereof.

TRAIL-R may be employed in inhibiting a biological activity of TRAIL, in *in vitro* or *in vivo* procedures. A purified TRAIL-R polypeptide may be used to inhibit binding of TRAIL to endogenous cell surface TRAIL-R. Biological effects that result from the binding of TRAIL to endogenous receptors thus are inhibited. Various forms of TRAIL-R may be employed, including, for example, the above-described TRAIL-R fragments, oligomers, derivatives, and variants that are capable of binding TRAIL. In one embodiment, a soluble TRAIL-R is employed to inhibit a biological activity of TRAIL, e.g., to inhibit TRAIL-mediated apoptosis of particular cells.

TRAIL-R may be administered to a mammal to treat a TRAIL-mediated disorder. Such TRAIL-mediated disorders include conditions caused (directly or indirectly) or exacerbated by TRAIL.

TRAIL-R may be useful for treating thrombotic microangiopathies. One such disorder is thrombotic thrombocytopenic purpura (TTP) (Kwaan, H.C., *Semin. Hematol.*, 24:71, 1987; Thompson et al., *Blood*, 80:1890, 1992). Increasing TTP-associated mortality rates have been reported by the U.S. Centers for Disease Control (Torok et al., *Am. J. Hematol.* 50:84, 1995).

Plasma from patients afflicted with TTP (including $HIV^+$ and $HIV^-$ patients) induces apoptosis of human endothelial cells of dermal microvascular origin, but not large vessel origin (Laurence et al., *Blood*, 87:3245, April 15, 1996). Plasma of TTP patients thus is thought to contain one or more factors that directly or indirectly induce apoptosis. As described in PCT application WO 97/01633 (hereby incorporated by reference), TRAIL is present in the serum of TTP patients, and may play a role in inducing apoptosis of microvascular endothelial cells.

Another thrombotic microangiopathy is hemolytic-uremic syndrome (HUS) (Moake, J.L., *Lancet*, 343:393, 1994; Melnyk et al., (*Arch. Intern. Med.*, 155:2077, 1995; Thompson et al., *supra*). One embodiment of the invention is directed to use of TRAIL-R to treat the condition that is often referred to as "adult HUS" (even though it can strike children as well). A disorder known as childhood/diarrhea-associated HUS differs in etiology from adult HUS.

Other conditions characterized by clotting of small blood vessels may be treated using TRAIL-R. Such conditions include but are not limited to the following. Cardiac problems seen in about 5-10% of pediatric AIDS patients are believed to involve clotting of small blood vessels. Breakdown of the microvasculature in the heart has been reported in multiple sclerosis patients. As a further example, treatment of systemic lupus erythematosus (SLE) is contemplated.

In one embodiment, a patient's blood or plasma is contacted with TRAIL-R *ex vivo*. The TRAIL-R may be bound to a suitable chromatography matrix by conventional procedures. The patient's blood or plasma flows through a chromatography column containing TRAIL-R bound to the matrix, before being returned to the patient. The immobilized receptor binds TRAIL, thus removing TRAIL protein from the patient's blood.

Alternatively, TRAIL-R may be administered *in vivo* to a patient afflicted with a thrombotic microangiopathy. In one embodiment, a soluble form of TRAIL-R is administered to the patient.

The present invention thus provides a method for treating a thrombotic microangiopathy, involving use of an effective amount of TRAIL-R. A TRAIL-R

polypeptide may be employed in *in vivo* or *ex vivo* procedures, to inhibit TRAIL-mediated damage to (e.g., apoptosis of) microvascular endothelial cells.

TRAIL-R may be employed in conjunction with other agents useful in treating a particular disorder. In an *in vitro* study reported by Laurence et al. (*Blood* 87:3245, 1996), some reduction of TTP plasma-mediated apoptosis of microvascular endothelial cells was achieved by using an anti-Fas blocking antibody, aurintricarboxylic acid, or normal plasma depleted of cryoprecipitate.

Thus, a patient may be treated with an agent that inhibits Fas-ligand-mediated apoptosis of endothelial cells, in combination with an agent that inhibits TRAIL-mediated apoptosis of endothelial cells. In one embodiment, TRAIL-R and an anti-FAS blocking antibody are both administered to a patient afflicted with a disorder characterized by thrombotic microangiopathy, such as TTP or HUS. Examples of blocking monoclonal antibodies directed against Fas antigen (CD95) are described in PCT application publication number WO 95/10540, hereby incorporated by reference.

Compositions comprising an effective amount of a TRAIL-R polypeptide of the present invention, in combination with other components such as a physiologically acceptable diluent, carrier, or excipient, are provided herein. TRAIL-R can be formulated according to known methods used to prepare pharmaceutically useful compositions. TRAIL-R can be combined in admixture, either as the sole active material or with other known active materials suitable for a given indication, with pharmaceutically acceptable diluents (e.g., saline, Tris-HCl, acetate, and phosphate buffered solutions), preservatives (e.g., thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable formulations for pharmaceutical compositions include those described in *Remington's Pharmaceutical Sciences*, 16th ed. 1980, Mack Publishing Company, Easton, PA.

In addition, such compositions can contain TRAIL-R complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of TRAIL-R, and are thus chosen according to the intended application. TRAIL-R expressed on the surface of a cell may find use, as well.

Compositions of the present invention may contain a TRAIL-R polypeptide in any form described herein, such as native proteins, variants, derivatives, oligomers, and biologically active fragments. In particular embodiments, the composition

comprises a soluble TRAIL-R polypeptide or an oligomer comprising soluble TRAIL-R polypeptides.

TRAIL-R can be administered in any suitable manner, e.g., topically, parenterally, or by inhalation. The term "parenteral" includes injection, e.g., by subcutaneous, intravenous, or intramuscular routes, also including localized administration, e.g., at a site of disease or injury. Sustained release from implants is also contemplated. One skilled in the pertinent art will recognize that suitable dosages will vary, depending upon such factors as the nature of the disorder to be treated, the patient's body weight, age, and general condition, and the route of administration. Preliminary doses can be determined according to animal tests, and the scaling of dosages for human administration are performed according to art-accepted practices.

Compositions comprising TRAIL-R nucleic acids in physiologically acceptable formulations are also contemplated. TRAIL-R DNA may be formulated for injection, for example.

Antibodies

Antibodies that are immunoreactive with TRAIL-R polypeptides are provided herein. Such antibodies specifically bind TRAIL-R, in that the antibodies bind to TRAIL-R *via* the antigen-binding sites of the antibody (as opposed to non-specific binding).

The TRAIL-R protein prepared as described in example 1 may be employed as an immunogen in producing antibodies immunoreactive therewith. Alternatively, another form of TRAIL-R, such as a fragment or fusion protein, is employed as the immunogen.

Polyclonal and monoclonal antibodies may be prepared by conventional techniques. See, for example, *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Kennet et al. (eds.), Plenum Press, New York (1980); and *Antibodies: A Laboratory Manual*, Harlow and Land (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1988). Production of monoclonal antibodies directed against TRAIL-R is further illustrated in example 4.

Antigen-binding fragments of such antibodies, which may be produced by conventional techniques, are also encompassed by the present invention. Examples of such fragments include, but are not limited to, Fab and $F(ab')_2$ fragments. Antibody fragments and derivatives produced by genetic engineering techniques are also provided.

The monoclonal antibodies of the present invention include chimeric antibodies, e.g., humanized versions of murine monoclonal antibodies. Such humanized antibodies may be prepared by known techniques, and offer the advantage of reduced immunogenicity when the antibodies are administered to humans. In one embodiment, a humanized monoclonal antibody comprises the variable region of a murine antibody (or just the antigen binding site thereof) and a constant region derived from a human antibody. Alternatively, a humanized antibody fragment may comprise the antigen binding site of a murine monoclonal antibody and a variable region fragment (lacking the antigen-binding site) derived from a human antibody. Procedures for the production of chimeric and further engineered monoclonal antibodies include those described in Riechmann et al. (*Nature 332*:323, 1988), Liu et al. (*PNAS 84*:3439, 1987), Larrick et al. (*Bio/Technology 7*:934, 1989), and Winter and Harris (*TIPS 14*:139, May, 1993).

Among the uses of the antibodies is use in assays to detect the presence of TRAIL-R polypeptides, either *in vitro* or *in vivo*. The antibodies also may be employed in purifying TRAIL-R proteins by immunoaffinity chromatography.

Those antibodies that additionally can block binding of TRAIL-R to TRAIL may be used to inhibit a biological activity that results from such binding. Such blocking antibodies may be identified using any suitable assay procedure, such as by testing antibodies for the ability to inhibit binding of TRAIL to cells expressing TRAIL-R. Examples of such cells are the Jurkat cells and PS1 cells described in example 2 below. Alternatively, blocking antibodies may be identified in assays for the ability to inhibit a biological effect that results from binding of TRAIL to target cells. Antibodies may be assayed for the ability to inhibit TRAIL-mediated lysis of Jurkat cells, for example.

Such an antibody may be employed in an *in vitro* procedure, or administered *in vivo* to inhibit a TRAIL-R-mediated biological activity. Disorders caused or exacerbated (directly or indirectly) by the interaction of TRAIL with cell surface TRAIL receptor thus may be treated. A therapeutic method involves *in vivo* administration of a blocking antibody to a mammal in an amount effective in inhibiting a TRAIL-mediated biological activity. Disorders caused or exacerbated by TRAIL, directly or indirectly, are thus treated. Monoclonal antibodies are generally preferred for use in such therapeutic methods. In one embodiment, an antigen-binding antibody fragment is employed.

A blocking antibody directed against TRAIL-R may be substituted for TRAIL-R in the above-described method of treating thrombotic microangiopathy, e.g., in treating

TTP or HUS. The antibody is administered *in vivo*, to inhibit TRAIL-mediated damage to (e.g., apoptosis of) microvascular endothelial cells.

Antibodies raised against TRAIL-R may be screened for agonistic (i.e., ligand-mimicking) properties. Such antibodies, upon binding to cell surface TRAIL-R, induce biological effects (e.g., transduction of biological signals) similar to the biological effects induced when TRAIL binds to cell surface TRAIL-R. Agonistic antibodies may be used to induce apoptosis of certain cancer cells or virally infected cells, as has been reported for TRAIL. The ability of TRAIL to kill cancer cells (including but not limited to leukemia, lymphoma, and melanoma cells) and virally infected cells is described in Wiley et al. (*Immunity* 3:673-682, 1995); and in PCT application WO 97/01633.

Compositions comprising an antibody that is directed against TRAIL-R, and a physiologically acceptable diluent, excipient, or carrier, are provided herein. Suitable components of such compositions are as described above for compositions containing TRAIL-R proteins.

Also provided herein are conjugates comprising a detectable (e.g., diagnostic) or therapeutic agent, attached to an antibody directed against TRAIL-R. Examples of such agents are presented above. The conjugates find use in *in vitro* or *in vivo* procedures.

<u>Nucleic Acids</u>

The present invention provides TRAIL-R nucleic acids. Such nucleic acids include, but are not limited to, DNA encoding the peptide described in example 2. Such DNAs can be identified from knowledge of the genetic code. Other nucleic acids of the present invention include isolated DNAs comprising the nucleotide sequence presented in SEQ ID NO:1 or SEQ ID NO:3.

The present invention provides isolated nucleic acids useful in the production of TRAIL-R polypeptides, e.g., in the recombinant expression systems discussed above. Such nucleic acids include, but are not limited to, the human TRAIL-R DNA of SEQ ID NO:1. Nucleic acid molecules of the present invention include TRAIL-R DNA in both single-stranded and double-stranded form, as well as the RNA complement thereof. TRAIL-R DNA includes, for example, cDNA, genomic DNA, chemically synthesized DNA, DNA amplified by PCR, and combinations thereof. Genomic DNA may be isolated by conventional techniques, e.g., using the cDNA of SEQ ID NO:1 or 3, or a suitable fragment thereof, as a probe.

DNAs encoding TRAIL-R in any of the forms contemplated herein (e.g., full length TRAIL-R or fragments thereof) are provided. Particular embodiments of

TRAIL-R-encoding DNAs include a DNA encoding the full length human TRAIL-R of SEQ ID NO:2 (including the N-terminal signal peptide), and a DNA encoding a full length mature human TRAIL-R. Other embodiments include DNA encoding a soluble TRAIL-R (e.g., encoding the extracellular domain of the protein of SEQ ID NO:2, either with or without the signal peptide).

One embodiment of the invention is directed to fragments of TRAIL-R nucleotide sequences comprising at least about 17 contiguous nucleotides of a TRAIL-R DNA sequence. In other embodiments, a DNA fragment comprises at least 30, or at least 60, contiguous nucleotides of a TRAIL-R DNA sequence. Nucleic acids provided herein include DNA and RNA complements of said fragments, along with both single-stranded and double-stranded forms of the TRAIL-R DNA.

Among the uses of TRAIL-R nucleic acid fragments is use as probes or primers. Using knowledge of the genetic code in combination with the amino acid sequences set forth in example 2, sets of degenerate oligonucleotides can be prepared. Such oligonucleotides find use as primers, e.g., in polymerase chain reactions (PCR), whereby TRAIL-R DNA fragments are isolated and amplified.

Other useful fragments of the TRAIL-R nucleic acids include antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target TRAIL-R mRNA (sense) or TRAIL-R DNA (antisense) sequences. Antisense or sense oligonucleotides, according to the present invention, comprise a fragment of the coding region of TRAIL-R DNA. Such a fragment generally comprises at least about 14 nucleotides, preferably from about 14 to about 30 nucleotides. The ability to derive an antisense or a sense oligonucleotide, based upon a cDNA sequence encoding a given protein is described in, for example, Stein and Cohen (*Cancer Res.* 48:2659, 1988) and van der Krol et al. (*BioTechniques* 6:958, 1988).

Binding of antisense or sense oligonucleotides to target nucleic acid sequences results in the formation of duplexes that block transcription or translation of the target sequence by one of several means, including enhanced degradation of the duplexes, premature termination of transcription or translation, or by other means. The antisense oligonucleotides thus may be used to block expression of TRAIL-R proteins. Antisense or sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester backbones (or other sugar linkages, such as those described in WO91/06629) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable *in vivo* (i.e., capable of

resisting enzymatic degradation) but retain sequence specificity to be able to bind to target nucleotide sequences.

Other examples of sense or antisense oligonucleotides include those oligonucleotides which are covalently linked to organic moieties, such as those described in WO 90/10448, and other moieties that increases affinity of the oligonucleotide for a target nucleic acid sequence, such as poly-(L-lysine). Further still, intercalating agents, such as ellipticine, and alkylating agents or metal complexes may be attached to sense or antisense oligonucleotides to modify binding specificities of the antisense or sense oligonucleotide for the target nucleotide sequence.

Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, $CaPO_4$-mediated DNA transfection, electroporation, or by using gene transfer vectors such as Epstein-Barr virus. In a preferred procedure, an antisense or sense oligonucleotide is inserted into a suitable retroviral vector. A cell containing the target nucleic acid sequence is contacted with the recombinant retroviral vector, either *in vivo* or *ex vivo*. Suitable retroviral vectors include, but are not limited to, those derived from the murine retrovirus M-MuLV, N2 (a retrovirus derived from M-MuLV), or the double copy vectors designated DCT5A, DCT5B and DCT5C (see WO 90/13641).

Sense or antisense oligonucleotides also may be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

Alternatively, a sense or an antisense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex, as described in WO 90/10448. The sense or antisense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase.

The following examples are provided to further illustrate particular embodiments of the invention, and are not to be construed as limiting the scope of the present invention.

## EXAMPLE 1: Purification of TRAIL-R Protein

A human TRAIL receptor (TRAIL-R) protein was prepared by the following procedure. Trail-R was isolated from the cell membranes of Jurkat cells, a human acute T leukemia cell line. Jurkat cells were chosen because a specific band can be affinity precipitated from surface-biotinylated Jurkat cells, using Flag®-TRAIL covalently coupled to affi-gel beads (Biorad Laboratories, Richmond, CA). The precipitated band has a molecular weight of about 52 kD. A minor specific band of about 42 kD also was present, possibly accounting for a proteolytic breakdown product or a less glycosylated form of TRAIL-R.

Approximately 50 billion Jurkat cells were harvested by centrifugation (80 ml of cell pellet), washed once with PBS, then shock frozen on liquid nitrogen. Plasma membranes were isolated according to method number three described in Maeda et al., *Biochim. et Biophys. Acta*, 731:115, 1983; hereby incorporated by reference) with five modifications:

1. The following protease inhibitors were included in all solutions at the indicated concentrations: Aprotinin, 150 nM; EDTA, 5 mM; Leupeptin, 1 μM; pA-PMSF, 20 μM; Pefabloc, 500 μM; Pepstatin A, 1 μM; PMSF, 500 μM.
2. Dithiothreitol was not used.
3. DNAase was not used in the homogenization solution.
4. 1.25 ml of homogenization buffer was used per ml of cell pellet.
5. The homogenization was accomplished by five passages through a ground glass dounce homogenizer.

After isolation of the cell membranes, proteins were solubilized by resuspending the isolated membranes in 50 ml PBS containing 1% octylglucoside and all of the above mentioned protease inhibitors at the above indicated concentrations. The resulting solution was then repeatedly vortexed during a thirty-minute incubation at 4°C. The solution was then centrifuged at 20,000 rpm in an SW28 rotor in an LE-80 Beckman ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) at 4°C for 30 minutes to obtain the supernatant (the membrane extract).

Chromatography

The first step of purification of TRAIL-R out of the membrane extract prepared above was affinity chromatography. The membrane extract was first applied to an anti-Flag® M2 affi-gel column (10 mg of monoclonal antibody M2 coupled to 2 ml of Affi-gel beads) to adsorb any nonspecifically binding material. The flow-through was then

applied to a Flag®-TRAIL affi-gel column (10 mg of recombinant protein coupled to 1 ml of affi-gel beads).

The Affi-gel support is an N-hydroxysuccinimide ester of a derivatized, crosslinked agarose gel bead (available from Biorad Laboratories, Richmond, CA). As discussed above, the Flag® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, provides an epitope reversibly bound by specific monoclonal antibodies, enabling rapid assay and facile purification of expressed recombinant protein. M2 is a monoclonal antibody that binds Flag®. Monoclonal antibodies that bind the Flag® peptide, as well as other reagents for preparing and using Flag® fusion proteins, are available from Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Connecticut. Preparation of Flag®-TRAIL fusion proteins (comprising Flag® fused to a soluble TRAIL polypeptide) is further described in PCT application WO 97/01633, hereby incorporated by reference.

The column was washed with 25 ml of each of the following buffers, in the order indicated:

1. PBS containing 1% octylglucoside
2. PBS
3. PBS containing an additional 200 mM NaCl
4. PBS

The bound material was eluted with 50 mM Na Citrate (pH 3) in 1 ml fractions and immediately neutralized with 300 µl of 1 M Tris-HCl (pH 8.5) per fraction. The TRAIL-binding activity of each fraction was determined by a TRAIL-R-specific ELISA as described below. Fractions with high TRAIL-binding activity were pooled, brought to 0.1 % Trifluoroacetic acid (TFA), and subsequently chromatographed on a capillary reversed-phase HPLC column [500 µm internal diameter X 25 cm fused silicone capillary column packed with 5 µm Vydac $C_4$ material (Vydac, Hesperia, CA)] using a linear gradient (2% per minute) from 0% to 100% acetonitrile in water containing 0.1% TFA. Fractions containing high TRAIL-binding activity are then determined as above, pooled, and, if desired, lyophilized.

<u>TRAIL-R-specific ELISA:</u>

Serial dilutions of TRAIL-R-containing samples (in 50 mM $NaHCO_3$, brought to pH 9 with NaOH) were coated onto Linbro/Titertek 96 well flat bottom E.I.A. microtitration plates (ICN Biomedicals Inc., Aurora, OH) at 100 µl/well. After incubation at 4°C for 16 hours, the wells were washed six times with 200 µl PBS containing 0.05% Tween-20 (PBS-Tween). The wells were then incubated with

Flag®-TRAIL at 1 μg/ml in PBS-Tween with 5% fetal calf serum (FCS) for 90 minutes (100 μl per well), followed by washing as above. Next, each well was incubated with the anti-Flag® monoclonal antibody M2 at 1 μg/ml in PBS-Tween containing 5% FCS for 90 minutes (100 μl per well), followed by washing as above. Subsequently, wells were incubated with a polyclonal goat anti-mIgG1-specific horseradish peroxidase-conjugated antibody (a 1:5000 dilution of the commercial stock in PBS-Tween containing 5% FCS) for 90 minutes (100 μl per well). The HRP-conjugated antibody was obtained from Southern Biotechnology Associates, Inc., Birmingham, Alabama. Wells then were washed six times, as above.

For development of the ELISA, a substrate mix [100 μl per well of a 1:1 premix of the TMB Peroxidase Substrate and Peroxidase Solution B (Kirkegaard Perry Laboratories, Gaithersburg, Maryland)] was added to the wells. After sufficient color reaction, the enzymatic reaction was terminated by addition of 2 N $H_2SO_4$ (50 μl per well). Color intensity (indicating TRAIL-binding activity) was determined by measuring extinction at 450 nm on a V Max plate reader (Molecular Devices, Sunnyvale, CA).

## EXAMPLE 2: Amino Acid Sequence

(a) TRAIL-R purified from Jurkat cells

TRAIL-R protein isolated from Jurkat cells was digested with trypsin, using conventional procedures. Amino acid sequence analysis was conducted on one of the peptide fragments produced by the tryptic digest. The fragment was found to contain the following sequence, which corresponds to amino acids 327 to 333 of the sequence presented in SEQ ID NO:2: VPANEGD.

(b) TRAIL-R purified from PS-1 cells

TRAIL-R protein was also isolated from PS-1 cells. PS-1 is a human B cell line that spontaneously arose after lethal irradiation of human peripheral blood lymphocytes (PBLs). The TRAIL-R protein was digested with trypsin, using conventional procedures. Amino acid sequence analysis was conducted on peptide fragments that resulted from the tryptic digest. One of the fragments was found to contain the following sequence, which, like the fragment presented in (a), corresponds to amino acids 327 to 333 of the sequence presented in SEQ ID NO:2: VPANEGD.

## EXAMPLE 3: DNA and Amino Acid Sequences

The amino acid sequence of additional tryptic digest peptide fragments of TRAIL-R was determined. Degenerate oligonucleotides, based upon the amino acid

sequence of two of the peptides, were prepared. A TRAIL-R DNA fragment was isolated and amplified by polymerase chain reaction (PCR), using the degenerate oligonucleotides as 5' and 3' primers. The PCR was conducted according to conventional procedures, using cDNA derived from the PS-1 cell line described in example 2 as the template. The nucleotide sequence of the isolated TRAIL-R DNA fragment (excluding portions corresponding to part of the primers), and the amino acid sequence encoded thereby, are presented in Figure 1 (SEQ ID NOS:3 and 4). The sequence of the entire TRAIL-R DNA fragment isolated by PCR corresponds to nucleotides 988 to 1164 of SEQ ID NO:1, which encode amino acids 330 to 388 of SEQ ID NO:2.

The amino acid sequence in SEQ ID NO:4 bears significant homology to the so-called death domains found in certain other receptors. The cytoplasmic region of Fas and TNF receptor type I each contain a death domain, which is associated with transduction of an apoptotic signal (Tartaglia et al. *Cell* 74:845, 1993; Itoh and Nagata, *J. Biol. Chem.* 268:10932, 1993). Thus, the sequence presented in SEQ ID NO:4 is believed to be found within the cytoplasmic domain of TRAIL-R.

A probe derived from the fragment isolated above was used to screen a cDNA library (human foreskin fibroblast-derived cDNA in λgt10 vector), and a human TRAIL-R cDNA was isolated. The nucleotide sequence of the coding region of this cDNA is presented in SEQ ID NO:1, and the amino acid sequence encoded thereby is shown in SEQ ID NO:2.

## EXAMPLE 4: Monoclonal Antibodies That Bind TRAIL-R

This example illustrates a method for preparing monoclonal antibodies that bind TRAIL-R. Suitable immunogens that may be employed in generating such antibodies include, but are not limited to, purified TRAIL-R protein or an immunogenic fragment thereof such as the extracellular domain, or fusion proteins containing TRAIL-R (e.g., a soluble TRAIL-R/Fc fusion protein).

Purified TRAIL-R can be used to generate monoclonal antibodies immunoreactive therewith, using conventional techniques such as those described in U.S. Patent 4,411,993. Briefly, mice are immunized with TRAIL-R immunogen emulsified in complete Freund's adjuvant, and injected in amounts ranging from 10-100 μg subcutaneously or intraperitoneally. Ten to twelve days later, the immunized animals are boosted with additional TRAIL-R emulsified in incomplete Freund's adjuvant. Mice are periodically boosted thereafter on a weekly to bi-weekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding

or tail-tip excision to test for TRAIL-R antibodies by dot blot assay, ELISA (Enzyme-Linked Immunosorbent Assay) or inhibition of TRAIL binding.

Following detection of an appropriate antibody titer, positive animals are provided one last intravenous injection of TRAIL-R in saline. Three to four days later, the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line, e.g., NS1 or preferably P3x63Ag8.653 (ATCC CRL 1580). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells are screened by ELISA for reactivity against purified TRAIL-R by adaptations of the techniques disclosed in Engvall et al., *Immunochem.* 8:871, 1971 and in U.S. Patent 4,703,004. A preferred screening technique is the antibody capture technique described in Beckmann et al., (*J. Immunol.* 144:4212, 1990) Positive hybridoma cells can be injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-TRAIL-R monoclonal antibodies. Alternatively, hybridoma cells can be grown *in vitro* in flasks or roller bottles by various techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to Protein A or Protein G can also be used, as can affinity chromatography based upon binding to TRAIL-R.

## EXAMPLE 5: Northern Blot Analysis

The tissue distribution of TRAIL-R mRNA was investigated by Northern blot analysis, as follows. An aliquot of a radiolabeled probe (the same radiolabeled probe used to screen the cDNA library in example 3) was added to two different human multiple tissue Northern blots (Clontech, Palo Alto, CA; Biochain, Palo Alto, CA). Hybridization was conducted overnight at 63°C in 50% formamide as previously described (March et al., *Nature* 315:641-647, 1985). The blots then were washed with 2X SSC, 0.1% SDS at 68°C for 30 minutes. A glycerol-aldehyde-phosphate dehydrogenase (GAPDH) specific probe was used to standardize for RNA loadings.

A single transcript of 4.4 kilobases (kb) was present in all tissues examined, including spleen, thymus, peripheral blood lymphocytes (PBLs), prostate, testis, ovary, uterus, placenta, and multiple tissues along the gastro-intestinal tract (including esophagus, stomach, duodenum, jejunum/ileum, colon, rectum, and small intestine). The cells and tissues with the highest levels of TRAIL-R mRNA are PBLs, spleen, and

ovary, as shown by comparison to control hybridizations with a GAPDH-specific probe.

## EXAMPLE 6: Binding Assay

Full length human TRAIL-R was expressed and tested for the ability to bind TRAIL. The binding assay was conducted as follows.

A fusion protein comprising a leucine zipper peptide fused to the N-terminus of a soluble TRAIL polypeptide (LZ-TRAIL) was employed in the assay. An expression construct was prepared, essentially as described for preparation of the Flag®-TRAIL expression construct in Wiley et al. (*Immunity*, 3:673-682, 1995; hereby incorporated by reference), except that DNA encoding the Flag® peptide was replaced with a sequence encoding a modified leucine zipper that allows for trimerization. The construct, in expression vector pDC409, encoded a leader sequence derived from human cytomegalovirus, followed by the leucine zipper moiety fused to the N-terminus of a soluble TRAIL polypeptide. The TRAIL polypeptide comprised amino acids 95-281 of human TRAIL (a fragment of the extracellular domain), as described in Wiley et al. (*supra*). The LZ-TRAIL was expressed in CHO cells, and purified from the culture supernatant.

The expression vector designated pDC409 is a mammalian expression vector derived from the pDC406 vector described in McMahan et al. (*EMBO J.* 10:2821-2832, 1991; hereby incorporated by reference). Features added to pDC409 (compared to pDC406) include additional unique restriction sites in the multiple cloning site (mcs); three stop codons (one in each reading frame) positioned downstream of the mcs; and a T7 polymerase promoter, downstream of the mcs, that faciliates sequencing of DNA inserted into the mcs.

For expression of full length human TRAIL-R protein, the entire coding region (i.e., the DNA sequence presented in SEQ ID NO:1) was amplified by polymerase chain reaction (PCR). The template employed in the PCR was the cDNA clone isolated from a human foreskin fibroblast cDNA library, as described in example 3. The isolated and amplified DNA was inserted into the expression vector pDC409, to yield a construct designated pDC409-TRAIL-R.

CrmA protein was employed to inhibit apoptosis of host cells expressing recombinant TRAIL-R, as discussed above and in example 8. An expression vector designated pDC409-CrmA contained DNA encoding poxvirus CrmA in pDC409. The 38-kilodalton cowpox-derived protein that was subsequently designated CrmA is

described in Pickup et al. (*Proc. Natl. Acad. Sci. USA* 83:7698-7702, 1986; hereby incorporated by reference).

CV-1/EBNA cells were co-transfected with pDC409-TRAIL-R together with pDC409-CrmA, or with pDC409-CrmA alone. The cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine. 48 hours after transfection, cells were detached non-enzymatically and incubated with LZ-TRAIL (5 μg/ml), a biotinylated anti-LZ monoclonal antibody (5 μg/ml), and phycoerythrin-conjugated streptavidin (1:400), before analysis by fluorescence-activated cell scanning (FACS). The cytometric analysis was conducted on a FACscan (Beckton Dickinson, San Jose, CA).

The CV-1/EBNA cells co-transfected with vectors encoding TRAIL-R and CrmA showed significantly enhanced binding of LZ-TRAIL, compared to the cells transfected with the CrmA-encoding vector alone.

## EXAMPLE 7: TRAIL-R Blocks TRAIL-Induced Apoptosis of Target Cells

TRAIL-R was tested for the ability to block TRAIL-induced apoptosis of Jurkat cells. The TRAIL-R employed in the assay was in the form of a fusion protein designated sTRAIL-R/Fc, which comprised the extracellular domain of human TRAIL-R, fused to the N-terminus of an Fc polypeptide derived from human IgG1.

CV1-EBNA cells were transfected with a recombinant expression vector comprising a gene fusion encoding the sTRAIL-R/Fc protein, in the pDC409 vector described in example 6, and cultured to allow expression of the fusion protein. The sTRAIL-R/Fc fusion protein was recovered from the culture supernatant. Procedures for fusing DNA encoding an IgG1 Fc polypeptide to DNA encoding a heterologous protein are described in Smith et al., (*Cell* 73:1349-1360, 1993); analogous procedures were employed herein.

A fusion protein designated TNF-R2-Fc, employed as a control, comprised the extracellular domain of the TNF receptor protein known as p75 or p80 TNF-R (Smith et al., *Science* 248:1019-1023, 1990; Smith et al. *Cell* 76:959-962, 1994), fused to an Fc polypeptide. A mouse monoclonal antibody that is a blocking antibody directed against human TRAIL, was employed in the assay as well.

Jurkat cells were incubated with varying or constant concentrations of LZ-TRAIL (the LZ-TRAIL fusion protein described in example 6), in the absence or presence of varying concentrations of sTRAIL-R-Fc, TNF-R2-Fc, or the TRAIL-specific monoclonal antibody. Cell death was quantitated using an MTT cell viability

assay (MTT is 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), as previously described (Mosmann, *J. Immunol. Methods* 65:55-63, 1983). The results are shown in Figure 2, which presents the percent cell death for Jurkat cells that were untreated (Δ) or were treated with varying (▲) or constant (○, ●, □, ■) concentrations of LZ-TRAIL (13 ng/ml) in the absence (●) or presence of varying concentrations of TRAIL-R2-Fc (■), TNF-R2-Fc (□), or the anti-TRAIL antibody (○). Varying concentrations for all substances started at 10 μg/ml and were serially diluted.

The anti-TRAIL monoclonal antibody and sTRAIL-R/Fc each blocked TRAIL-induced apoptosis in a dose dependent fashion, whereas TNFR2-Fc did not. Thus, the extracellular domain of TRAIL-R is capable of binding to TRAIL and inhibiting TRAIL-mediated apoptosis of target cells.

## EXAMPLE 8: TRAIL-R-induced apoptosis is blocked by caspase inhibitors and FADD-DN

CV-1/EBNA cells were transfected, by the DEAE-dextran method, with expression plasmids for TRAIL-R (pDC409-TRAIL-R), together with a threefold excess of empty expression vector (pDC409) in the presence or absence of z-VAD-fmk (10μM; in the culture medium), or together with a threefold excess of expression vector pDC409-CrmA, pDC409-p35, or pDC409-FADD-DN. In addition, 400 ng/slide of an expression vector for the *E. coli lacz* gene was co-transfected together with all DNA mixes. The transfected cells were cultured in chambers mounted on slides.

The mammalian expression vector pDC409, and the pDC409-TRAIL-R vector encoding full length human TRAIL-R, are described in example 6. The tripeptide derivative zVAD-fmk (benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone) is available from Enzyme System Products, Dublin, California.

The 38-kilodalton cowpox-derived protein that was subsequently designated CrmA is described in Pickup et al. (*Proc. Natl. Acad. Sci. USA* 83:7698-7702, 1986; hereby incorporated by reference). Sequence information for CrmA is presented in Figure 4 of Pickup et al., *supra*.

A 35-kilodalton protein encoded by *Autographa californica* nuclear polyhedrosis virus, a baculovirus, is described in Friesen and Miller (*J. Virol.* 61:2264-2272, 1987; hereby incorporated by reference). Sequence information for this protein, designated baculovirus p35 herein, is presented in Figure 5 of Friesen and Miller, *supra*.

FADD (also designated MORT1) is described in Boldin et al. (*J. Biol. Chem.* 270:7795-7798, 1995; hereby incorporated by reference). The protein referred to as

FADD-DN (FADD dominant negative) is a C-terminal fragment of FADD that includes the death domain. DNA encoding FADD-DN, fused to an N-terminal Flag® epitope tag (described above), was inserted into the pDC409 expression vector described in example 6, to form pDC409-FADD-DN. The FADD-DN polypeptide corresponds to amino acids 117 through 245 of the MORT1 amino acid sequence presented in Boldin et al., *supra*.

48 hours after transfection, cells were washed with PBS, fixed with glutaraldehyde and incubated with X-gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside). Cells expressing β-galactosidase stain blue. A decrease in the percentage of stained cells indicates loss of β-galactosidase expression, and correlates with death of cells that express the protein(s) co-transfected with the *lacz* gene.

The results are presented in Figure 3, wherein the values plotted represent the mean and standard deviation of at least three separate experiments. Poxvirus CrmA, baculovirus p35, FADD-DN, and z-VAD-fmk each effectively reduced death of transfected cells expressing TRAIL-R.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Rauch, Charles
Walczak, Henning

(ii) TITLE OF INVENTION: Receptor That Binds TRAIL

(iii) NUMBER OF SEQUENCES: 4

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Power Macintosh
(C) OPERATING SYSTEM: Macintosh 7.6
(D) SOFTWARE: Microsoft Word, Version 6.0.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US --to be assigned--
(B) FILING DATE: 04-JUN-1997
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/829,536
(B) FILING DATE: 28-MAR-1997

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/815,255
(B) FILING DATE: 12-MAR-1997

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/799,861
(B) FILING DATE: 13-FEB-1997

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1323 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: huTrail-R

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1323

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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 1               5                  10                  15

AGG CAC GGC CCA GGA CCC AGG GAG GCG CGG GGA GCC AGG CCT GGG CCC      96
Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro Gly Pro
            20                  25                  30

CGG GTC CCC AAG ACC CTT GTG CTC GTT GTC GCC GCG GTC CTG CTG TTG     144
Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val Leu Leu Leu
        35                  40                  45

GTC TCA GCT GAG TCT GCT CTG ATC ACC CAA CAA GAC CTA GCT CCC CAG     192
Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln
    50                  55                  60

CAG AGA GCG GCC CCA CAA CAA AAG AGG TCC AGC CCC TCA GAG GGA TTG     240
Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu
65                  70                  75                  80

TGT CCA CCT GGA CAC CAT ATC TCA GAA GAC GGT AGA GAT TGC ATC TCC     288
Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp Cys Ile Ser
                85                  90                  95

TGC AAA TAT GGA CAG GAC TAT AGC ACT CAC TGG AAT GAC CTC CTT TTC     336
Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His Trp Asn Asp Leu Leu Phe
            100                 105                 110

TGC TTG CGC TGC ACC AGG TGT GAT TCA GGT GAA GTG GAG CTA AGT CCG     384
Cys Leu Arg Cys Thr Arg Cys Asp Ser Gly Glu Val Glu Leu Ser Pro
        115                 120                 125

TGC ACC ACG ACC AGA AAC ACA GTG TGT CAG TGC GAA GAA GGC ACC TTC     432
Cys Thr Thr Thr Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe
    130                 135                 140

CGG GAA GAA GAT TCT CCT GAG ATG TGC CGG AAG TGC CGC ACA GGG TGT     480
Arg Glu Glu Asp Ser Pro Glu Met Cys Arg Lys Cys Arg Thr Gly Cys
145                 150                 155                 160
```

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Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile
165 170 175

GAA TGT GTC CAC AAA GAA TCA GGT ACA AAG CAC AGT GGG GAA GCC CCA 576
Glu Cys Val His Lys Glu Ser Gly Thr Lys His Ser Gly Glu Ala Pro
180 185 190

GCT GTG GAG GAG ACG GTG ACC TCC AGC CCA GGG ACT CCT GCC TCT CCC 624
Ala Val Glu Glu Thr Val Thr Ser Ser Pro Gly Thr Pro Ala Ser Pro
195 200 205

TGT TCT CTC TCA GGC ATC ATC ATA GGA GTC ACA GTT GCA GCC GTA GTC 672
Cys Ser Leu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val
210 215 220

TTG ATT GTG GCT GTG TTT GTT TGC AAG TCT TTA CTG TGG AAG AAA GTC 720
Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys Val
225 230 235 240

CTT CCT TAC CTG AAA GGC ATC TGC TCA GGT GGT GGT GGG GAC CCT GAG 768
Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp Pro Glu
245 250 255

CGT GTG GAC AGA AGC TCA CAA CGA CCT GGG GCT GAG GAC AAT GTC CTC 816
Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp Asn Val Leu
260 265 270

AAT GAG ATC GTG AGT ATC TTG CAG CCC ACC CAG GTC CCT GAG CAG GAA 864
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275 280 285

ATG GAA GTC CAG GAG CCA GCA GAG CCA ACA GGT GTC AAC ATG TTG TCC 912
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290 295 300

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305 310 315 320

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325 330 335

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340 345 350

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355 360 365

AAG GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG TAC ACG 1152
Lys Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu Tyr Thr
370 375 380

ATG CTG ATA AAG TGG GTC AAC AAA ACC GGG CGA GAT GCC TCT GTC CAC 1200
Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg Asp Ala Ser Val His
385 390 395 400

ACC CTG CTG GAT GCC TTG GAG ACG CTG GGA GAG AGA CTT GCC AAG CAG 1248
Thr Leu Leu Asp Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln
405 410 415

AAG ATT GAG GAC CAC TTG TTG AGC TCT GGA AAG TTC ATG TAT CTA GAA 1296
Lys Ile Glu Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu
420 425 430

GGT AAT GCA GAC TCT GCC ATG TCC TAA 1323
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435 440

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 440 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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20 25 30

Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val Leu Leu Leu
35 40 45

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50 55 60

Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu
65 70 75 80

Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp Cys Ile Ser
85 90 95

Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His Trp Asn Asp Leu Leu Phe
100 105 110

Cys Leu Arg Cys Thr Arg Cys Asp Ser Gly Glu Val Glu Leu Ser Pro
115 120 125

Cys Thr Thr Thr Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe
130 135 140

Arg Glu Glu Asp Ser Pro Glu Met Cys Arg Lys Cys Arg Thr Gly Cys
145 150 155 160

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165 170 175

Glu Cys Val His Lys Glu Ser Gly Thr Lys His Ser Gly Glu Ala Pro
180 185 190

Ala Val Glu Glu Thr Val Thr Ser Ser Pro Gly Thr Pro Ala Ser Pro
195 200 205

Cys Ser Leu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val
210 215 220

Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys Val
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Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp Pro Glu
245 250 255

Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp Asn Val Leu
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Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val Pro Glu Gln Glu
275 280 285

Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly Val Asn Met Leu Ser
290 295 300

Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala Glu Ala Glu Arg Ser
305 310 315 320

Gln Arg Arg Arg Leu Leu Val Pro Ala Asn Glu Gly Asp Pro Thr Glu
325 330 335

Thr Leu Arg Gln Cys Phe Asp Asp Phe Ala Asp Leu Val Pro Phe Asp
340 345 350

Ser Trp Glu Pro Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu Ile
355 360 365

Lys Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu Tyr Thr
370 375 380

Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg Asp Ala Ser Val His
385 390 395 400

Thr Leu Leu Asp Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln
405 410 415

Lys Ile Glu Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu
420 425 430

Gly Asn Ala Asp Ser Ala Met Ser *
435 440

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:
(B) CLONE: huTrail-R frag

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..155

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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    1               5                  10                  15

TTT GAC TCC TGG GAG CCG CTC ATG AGG AAG TTG GGC CTC ATG GAC AAT     95
Phe Asp Ser Trp Glu Pro Leu Met Arg Lys Leu Gly Leu Met Asp Asn
                20                  25                  30

GAG ATA AAG GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG    143
Glu Ile Lys Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu
            35                  40                  45

TNC ACN ATG CTG AT                                                 157
Xaa Thr Met Leu
        50
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Glu Thr Leu Arg Gln Cys Phe Asp Asp Phe Ala Asp Leu Val Pro Phe
 1               5                  10                  15
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Asp Ser Trp Glu Pro Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu
20 25 30

Ile Lys Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu Xaa
35 40 45

Thr Met Leu
50

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: FLAG peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asp Tyr Lys Asp Asp Asp Asp Lys
1 5

What is claimed is:

1. A purified TRAIL receptor (TRAIL-R) polypeptide that is capable of binding TRAIL, wherein the TRAIL-R is characterized as comprising the amino acid sequence VPANEGD.

2. A TRAIL-R polypeptide of claim 1, wherein said polypeptide is further characterized by a molecular weight of about 50 to 55 kilodaltons.

3. A TRAIL-R polypeptide of claim 1, wherein said polypeptide is further characterized by comprising the amino acid sequence ETLRQCFDDFADLVPFDS WEPLMRKLGLMDNEIKVAKAEAAGHRDTLXTML.

4. A TRAIL-R polypeptide of claim 3, wherein said polypeptide is further characterized by a molecular weight of about 50 to 55 kilodaltons.

5. A purified TRAIL-R polypeptide selected from the group consisting of:

a) the TRAIL-R polypeptide of SEQ ID NO:2; and

b) a fragment of the polypeptide of (a), wherein said fragment is capable of binding TRAIL.

6. A TRAIL-R polypeptide of claim 5, wherein said polypeptide comprises amino acids x to 440 of SEQ ID NO:2, wherein x represents an integer from 51 through 59.

7. A TRAIL-R polypeptide of claim 6, wherein said polypeptide comprises amino acids 54 to 440 of SEQ ID NO:2.

8. A TRAIL-R polypeptide of claim 5, wherein said fragment is a soluble TRAIL-R comprising the extracellular domain of the TRAIL-R protein of SEQ ID NO:2.

9. A purified TRAIL-R polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence presented in SEQ ID NO:2.

10. A TRAIL-R polypeptide of claim 9, wherein said polypeptide comprises an amino acid sequence that is at least 90% identical to the amino acid sequence presented in SEQ ID NO:2.

11. A TRAIL-R polypeptide of claim 10, wherein said polypeptide comprises an amino acid sequence that is at least 95% identical to the amino acid sequence presented in SEQ ID NO:2.

12. A TRAIL-R polypeptide of claim 9, wherein said polypeptide is naturally occurring.

13. An oligomer comprising from two to four TRAIL-R polypeptides of claim 5.

14. An oligomer comprising from two to four TRAIL-R polypeptides of claim 8.

15. A composition comprising a TRAIL-R polypeptide of claim 5, and a physiologically acceptable diluent, excipient, or carrier.

16. A composition comprising a TRAIL-R polypeptide of claim 8, and a physiologically acceptable diluent, excipient, or carrier.

17. An isolated TRAIL-R DNA, wherein said DNA comprises the nucleotide sequence presented in Figure 1.

18. An isolated TRAIL-R DNA, wherein said DNA encodes a polypeptide selected from the group consisting of:

a) the TRAIL-R polypeptide of SEQ ID NO:2; and

b) a fragment of the polypeptide of (a), wherein said fragment is capable of binding TRAIL.

19. A TRAIL-R DNA of claim 18, wherein said DNA encodes amino acids 1 to 440 of SEQ ID NO:2.

20. A TRAIL-R DNA of claim 18, wherein said polypeptide comprises amino acids x to 440 of SEQ ID NO:2, wherein x represents an integer from 51 through 59.

21. A TRAIL-R DNA of claim 20, wherein said polypeptide comprises amino acids 54 to 440 of SEQ ID NO:2.

22. A TRAIL-R DNA of claim 18, wherein said fragment is a soluble TRAIL-R comprising the extracellular domain of the TRAIL-R protein of SEQ ID NO:2.

23. An isolated TRAIL-R DNA, wherein said DNA encodes a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence presented in SEQ ID NO:2.

24. A TRAIL-R DNA of claim 23, wherein said polypeptide comprises an amino acid sequence that is at least 90% identical to the amino acid sequence presented in SEQ ID NO:2.

25. A TRAIL-R DNA of claim 24, wherein said polypeptide comprises an amino acid sequence that is at least 95% identical to the amino acid sequence presented in SEQ ID NO:2.

26. A TRAIL-R DNA of claim 23, wherein said polypeptide is naturally occurring.

27. An expression vector comprising a DNA according to claim 18.

28. An expression vector comprising a DNA according to claim 19.

29. An expression vector comprising a DNA according to claim 20.

30. An expression vector comprising a DNA according to claim 22.

31. An expression vector comprising a DNA according to claim 23.

32. A host cell transformed with an expression vector of claim 27.

33. A host cell transformed with an expression vector of claim 28.

34. A host cell transformed with an expression vector of claim 29.

35. A host cell transformed with an expression vector of claim 30.

36. A host cell transformed with an expression vector of claim 31.

37. An isolated TRAIL-R DNA comprising at least 60 nucleotides of the sequence of SEQ ID NO:1, or the DNA or RNA complement thereof.

38. An antibody that is directed against a TRAIL-R polypeptide of claim 5, or an antigen-binding fragment of said antibody.

39. An antibody of claim 38, wherein the antibody is a monoclonal antibody.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Rauch, Charles
Walczak, Henning

(ii) TITLE OF INVENTION: Receptor That Binds TRAIL

(iii) NUMBER OF SEQUENCES: 4

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Power Macintosh
(C) OPERATING SYSTEM: Macintosh 7.6
(D) SOFTWARE: Microsoft Word, Version 6.0.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US --to be assigned--
(B) FILING DATE: 04-JUN-1997
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/829,536
(B) FILING DATE: 28-MAR-1997

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/815,255
(B) FILING DATE: 12-MAR-1997

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/799,861
(B) FILING DATE: 13-FEB-1997

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(C) TELEX: 756822

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1323 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: huTrail-R

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1323

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATG GAA CAA CGG GGA CAG AAC GCC CCG GCC GCT TCG GGG GCC CGG AAA      48
Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg Lys
  1               5                  10                  15

AGG CAC GGC CCA GGA CCC AGG GAG GCG CGG GGA GCC AGG CCT GGG CCC      96
Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro Gly Pro
             20                  25                  30

CGG GTC CCC AAG ACC CTT GTG CTC GTT GTC GCC GCG GTC CTG CTG TTG     144
Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val Leu Leu Leu
         35                  40                  45

GTC TCA GCT GAG TCT GCT CTG ATC ACC CAA CAA GAC CTA GCT CCC CAG     192
Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln
     50                  55                  60

CAG AGA GCG GCC CCA CAA CAA AAG AGG TCC AGC CCC TCA GAG GGA TTG     240
Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu
 65                  70                  75                  80

TGT CCA CCT GGA CAC CAT ATC TCA GAA GAC GGT AGA GAT TGC ATC TCC     288
Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp Cys Ile Ser
                 85                  90                  95

TGC AAA TAT GGA CAG GAC TAT AGC ACT CAC TGG AAT GAC CTC CTT TTC     336
Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His Trp Asn Asp Leu Leu Phe
            100                 105                 110

TGC TTG CGC TGC ACC AGG TGT GAT TCA GGT GAA GTG GAG CTA AGT CCG     384
Cys Leu Arg Cys Thr Arg Cys Asp Ser Gly Glu Val Glu Leu Ser Pro
        115                 120                 125

TGC ACC ACG ACC AGA AAC ACA GTG TGT CAG TGC GAA GAA GGC ACC TTC     432
Cys Thr Thr Thr Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe
    130                 135                 140

CGG GAA GAA GAT TCT CCT GAG ATG TGC CGG AAG TGC CGC ACA GGG TGT     480
Arg Glu Glu Asp Ser Pro Glu Met Cys Arg Lys Cys Arg Thr Gly Cys
145                 150                 155                 160
```

```
CCC AGA GGG ATG GTC AAG GTC GGT GAT TGT ACA CCC TGG AGT GAC ATC     528
Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile
                165                 170                 175

GAA TGT GTC CAC AAA GAA TCA GGT ACA AAG CAC AGT GGG GAA GCC CCA     576
Glu Cys Val His Lys Glu Ser Gly Thr Lys His Ser Gly Glu Ala Pro
            180                 185                 190

GCT GTG GAG GAG ACG GTG ACC TCC AGC CCA GGG ACT CCT GCC TCT CCC     624
Ala Val Glu Glu Thr Val Thr Ser Ser Pro Gly Thr Pro Ala Ser Pro
        195                 200                 205

TGT TCT CTC TCA GGC ATC ATC ATA GGA GTC ACA GTT GCA GCC GTA GTC     672
Cys Ser Leu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val
    210                 215                 220

TTG ATT GTG GCT GTG TTT GTT TGC AAG TCT TTA CTG TGG AAG AAA GTC     720
Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys Val
225                 230                 235                 240

CTT CCT TAC CTG AAA GGC ATC TGC TCA GGT GGT GGT GGG GAC CCT GAG     768
Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp Pro Glu
                245                 250                 255

CGT GTG GAC AGA AGC TCA CAA CGA CCT GGG GCT GAG GAC AAT GTC CTC     816
Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp Asn Val Leu
            260                 265                 270

AAT GAG ATC GTG AGT ATC TTG CAG CCC ACC CAG GTC CCT GAG CAG GAA     864
Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val Pro Glu Gln Glu
        275                 280                 285

ATG GAA GTC CAG GAG CCA GCA GAG CCA ACA GGT GTC AAC ATG TTG TCC     912
Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly Val Asn Met Leu Ser
    290                 295                 300

CCC GGG GAG TCA GAG CAT CTG CTG GAA CCG GCA GAA GCT GAA AGG TCT     960
Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala Glu Ala Glu Arg Ser
305                 310                 315                 320

CAG AGG AGG AGG CTG CTG GTT CCA GCA AAT GAA GGT GAT CCC ACT GAG    1008
Gln Arg Arg Arg Leu Leu Val Pro Ala Asn Glu Gly Asp Pro Thr Glu
                325                 330                 335

ACT CTG AGA CAG TGC TTC GAT GAC TTT GCA GAC TTG GTG CCC TTT GAC    1056
Thr Leu Arg Gln Cys Phe Asp Asp Phe Ala Asp Leu Val Pro Phe Asp
            340                 345                 350

TCC TGG GAG CCG CTC ATG AGG AAG TTG GGC CTC ATG GAC AAT GAG ATA    1104
Ser Trp Glu Pro Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu Ile
        355                 360                 365

AAG GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG TAC ACG    1152
Lys Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu Tyr Thr
    370                 375                 380
```

ATG CTG ATA AAG TGG GTC AAC AAA ACC GGG CGA GAT GCC TCT GTC CAC 1200
Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg Asp Ala Ser Val His
385 390 395 400

ACC CTG CTG GAT GCC TTG GAG ACG CTG GGA GAG AGA CTT GCC AAG CAG 1248
Thr Leu Leu Asp Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln
405 410 415

AAG ATT GAG GAC CAC TTG TTG AGC TCT GGA AAG TTC ATG TAT CTA GAA 1296
Lys Ile Glu Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu
420 425 430

GGT AAT GCA GAC TCT GCC ATG TCC TAA 1323
Gly Asn Ala Asp Ser Ala Met Ser *
435 440

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 440 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg Lys
1 5 10 15

Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro Gly Pro
20 25 30

Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val Leu Leu Leu
35 40 45

Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln
50 55 60

Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu
65 70 75 80

Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp Cys Ile Ser
85 90 95

Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His Trp Asn Asp Leu Leu Phe
100 105 110

Cys Leu Arg Cys Thr Arg Cys Asp Ser Gly Glu Val Glu Leu Ser Pro
115 120 125

Cys Thr Thr Thr Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe
130 135 140

Arg Glu Glu Asp Ser Pro Glu Met Cys Arg Lys Cys Arg Thr Gly Cys
145 150 155 160

Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile
165 170 175

Glu Cys Val His Lys Glu Ser Gly Thr Lys His Ser Gly Glu Ala Pro
180 185 190

Ala Val Glu Glu Thr Val Thr Ser Ser Pro Gly Thr Pro Ala Ser Pro
195 200 205

Cys Ser Leu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val
210 215 220

Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys Val
225 230 235 240

Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp Pro Glu
245 250 255

Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp Asn Val Leu
260 265 270

Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val Pro Glu Gln Glu
275 280 285

Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly Val Asn Met Leu Ser
290 295 300

Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala Glu Ala Glu Arg Ser
305 310 315 320

Gln Arg Arg Arg Leu Leu Val Pro Ala Asn Glu Gly Asp Pro Thr Glu
325 330 335

Thr Leu Arg Gln Cys Phe Asp Asp Phe Ala Asp Leu Val Pro Phe Asp
340 345 350

Ser Trp Glu Pro Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu Ile
355 360 365

Lys Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu Tyr Thr
370 375 380

Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg Asp Ala Ser Val His
385 390 395 400

Thr Leu Leu Asp Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln
405 410 415

Lys Ile Glu Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu
420 425 430

Gly Asn Ala Asp Ser Ala Met Ser *
435 440

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 157 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vii) IMMEDIATE SOURCE:
(B) CLONE: huTrail-R frag

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..155

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
CT GAG ACT CTG AGA CAG TGC TTC GAT GAC TTT GCA GAC TTG GTG CCC      47
   Glu Thr Leu Arg Gln Cys Phe Asp Asp Phe Ala Asp Leu Val Pro
    1               5                  10                  15

TTT GAC TCC TGG GAG CCG CTC ATG AGG AAG TTG GGC CTC ATG GAC AAT     95
Phe Asp Ser Trp Glu Pro Leu Met Arg Lys Leu Gly Leu Met Asp Asn
                 20                  25                  30

GAG ATA AAG GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG    143
Glu Ile Lys Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu
             35                  40                  45

TNC ACN ATG CTG AT                                                 157
Xaa Thr Met Leu
         50
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Glu Thr Leu Arg Gln Cys Phe Asp Asp Phe Ala Asp Leu Val Pro Phe
 1               5                  10                  15
```

Asp Ser Trp Glu Pro Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu
20 25 30

Ile Lys Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu Xaa
35 40 45

Thr Met Leu
50

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: FLAG peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asp Tyr Lys Asp Asp Asp Asp Lys
1 5

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**AS ORIGINALLY FILED**

# FIGURE 1

```
    CTGAGACTCTGAGACAGTGCTTTGATGACTTTGCAGACTTGGTGCCCTTTGACTCCTGGG
1   ---------+---------+---------+---------+---------+---------+ 60
    GACTCTGAGACTCTGTCACGAAACTACTGAAACGTCTGAACCACGGGAAACTGAGGACCC

     E  T  L  R  Q  C  F  D  D  F  A  D  L  V  P  F  D  S  W  E -

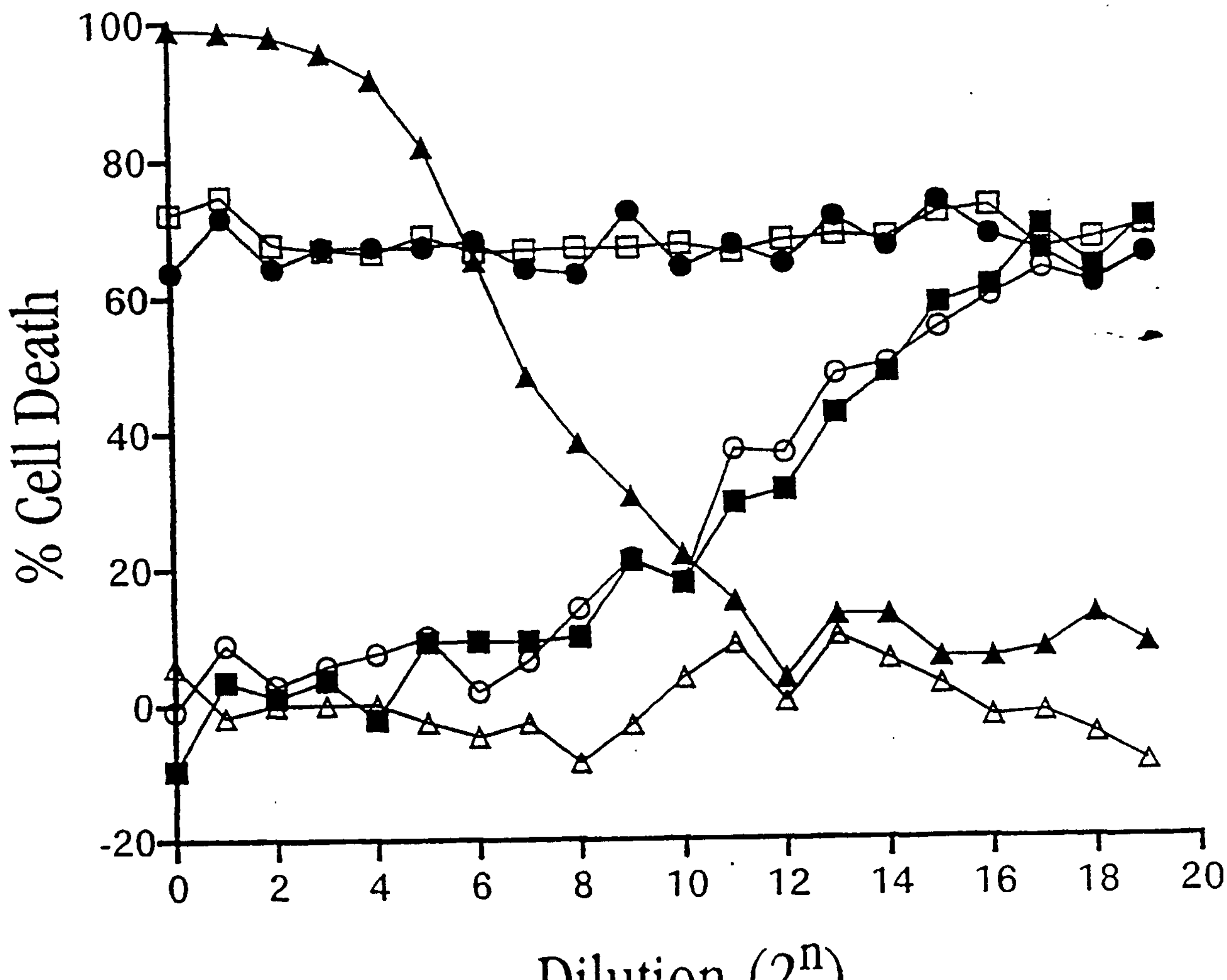
    AGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGG
61  ---------+---------+---------+---------+---------+---------+ 120
    TCGGCGAGTACTCCTTCAACCCGGAGTACCTGTTACTCTATTTCCACCGATTTCGACTCC

     P  L  M  R  K  L  G  L  M  D  N  E  I  K  V  A  K  A  E  A -

    CAGCGGGCCACAGGGACACCTTGTNCACNATGCTGAT
121 ---------+---------+---------+------- 157
    GTCGCCCGGTGTCCCTGTGGAACANGTGNTACGACTA

     A  G  H  R  D  T  L  X  T  M  L     -
```

FIGURE 2


100
80
60
40
20
0
-20
0
2
4
6
8
10
12
14
16
18
20
% Cell Death
Dilution (2^n)

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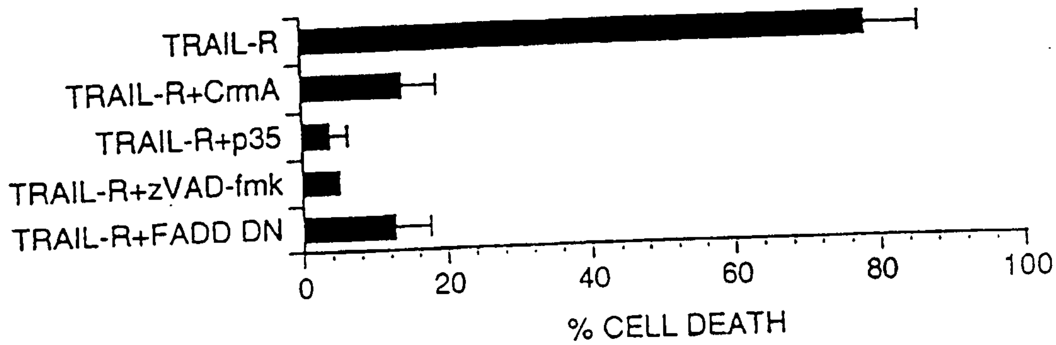
AS ORIGINALLY FILED

FIGURE 3



# EXHIBIT D

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Express Mail Certificate no. EM317869553US

Immunex Corporation 2625-B

## TITLE

# RECEPTOR THAT BINDS TRAIL

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application serial no. __________, filed March 12, 1997, currently pending, which is a continuation-in-part of application serial no. 08/799,861, filed February 13, 1997, currently pending.

## BACKGROUND OF THE INVENTION

A protein known as TNF-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family of ligands (Wiley et al., *Immunity*, 3:673-682, 1995). TRAIL has demonstrated the ability to induce apoptosis of certain transformed cells, including a number of different types of cancer cells as well as virally infected cells (PCT application WO 97/01633 and Wiley et al., *supra*).

Identification of receptor protein(s) that bind TRAIL would prove useful in further study of the biological activities of TRAIL. However, prior to the present invention, no receptor for TRAIL had been reported.

## SUMMARY OF THE INVENTION

The present invention is directed to a novel protein designated TRAIL receptor (TRAIL-R), which binds to a protein known as TNF-related apoptosis-inducing ligand (TRAIL). DNA encoding TRAIL-R, and expression vectors comprising such DNA, are provided. A method for producing TRAIL-R polypeptides comprises culturing host cells transformed with a recombinant expression vector encoding TRAIL-R, under conditions that promote expression of TRAIL-R, then recovering the expressed TRAIL-R polypeptides from the culture. Antibodies that are immunoreactive with TRAIL-R are also provided.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 presents the nucleotide sequence of a human TRAIL receptor DNA fragment, as well as the amino acid sequence encoded thereby. This DNA fragment was isolated as described in Example 3.

Figure 2 presents the nucleotide sequence of the coding region of a human TRAIL receptor DNA. An initiation codon (ATG) and termination codon (TAA) are underlined.

Figure 3 presents the amino acid sequence encoded by the nucleotide sequence of Figure 2.

## DETAILED DESCRIPTION OF THE INVENTION

A novel protein designated TRAIL receptor (TRAIL-R) is provided herein. TRAIL-R binds to the cytokine designated TNF-related apoptosis-inducing ligand (TRAIL). Certain uses of TRAIL-R flow from this ability to bind TRAIL, as discussed further below. TRAIL-R finds use in inhibiting biological activities of TRAIL, or in purifying TRAIL by affinity chromatography, for example.

TRAIL-R protein or immunogenic fragments thereof may be employed as immunogens to generate antibodies that are immunoreactive therewith. In one embodiment of the invention, the antibodies are monoclonal antibodies.

A human TRAIL-R protein was purified as described in example 1. In example 2, amino acid sequence information derived from fragments of TRAIL-R is presented. One embodiment of the invention is directed to a purified human TRAIL-R protein that is capable of binding TRAIL, wherein the TRAIL-R is characterized as comprising the amino acid sequence VPANEGD. In another embodiment, the TRAIL-R additionally comprises the sequence ETLRQCFDDFADLVPFDSWEPLMRKLGLMDNEIKVAKA EAAGHRDTLXTML. Also provided are TRAIL-R fragments comprising one of the characterizing amino acid sequences.

The nucleotide sequence of a TRAIL-R DNA fragment, and the amino acid sequence encoded thereby, are presented in Figure 1. This DNA fragment was isolated by polymerase chain reaction, as described in example 3. The amino acid sequence presented in Figure 1 has characteristics of the so-called "death domains" found in the cytoplasmic region of certain other receptor proteins. Such domains have been reported to be associated with transduction of apoptotic signals. The cytoplasmic region of Fas and TNF receptor type I each contain a death domain (Tartaglia et al. *Cell* 74:845, 1993; Itoh and Nagata, *J. Biol. Chem.* 268:10932, 1993).

Figure 2 presents the nucleotide sequence of the coding region of a human TRAIL receptor DNA. This sequence includes an initiation codon (ATG) and a termination codon (TAA), which are underlined. The amino acid sequence encoded by the DNA sequence of Figure 2 is shown in Figure 3. The fragment depicted in Figure 1 corresponds to the region of TRAIL-R that is presented as amino acids 336 to 386 in Figure 3.

The TRAIL-R protein of Figure 3 includes an N-terminal hydrophobic region that functions as a signal peptide, followed by an extracellular domain, a transmembrane region comprising amino acids 211 through 231, and a C-terminal cytoplasmic domain. Computer analysis predicts that the signal peptide corresponds to residues 1 through 51 of Figure 3. Cleavage of the signal peptide thus would yield a mature protein comprising amino acids 52 through 440 of Figure 3. The calculated molecular weight for a mature protein containing residues 52 to 440 of Figure 3 is about 43 kilodaltons.

The skilled artisan will recognize that the molecular weight of particular preparations of TRAIL-R protein may differ, according to such factors as the degree of glycosylation. The glycosylation pattern of a particular preparation of TRAIL-R may vary according to the type of cells in which the protein is expressed, for example. Further, a given preparation may include multiple differentially glycosylated species of the protein. TRAIL-R polypeptides with or without associated native-pattern glycosylation are provided herein.

In one embodiment, the protein is characterized by a molecular weight within the range of about 50 to 55 kilodaltons, which is the molecular weight determined for a preparation of native, full length, human TRAIL-R. Molecular weight can be determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

As used herein, the term "TRAIL-R" refers to a genus of polypeptides that are substantially homologous to the human TRAIL-R protein isolated in example 1. The present invention encompasses TRAIL-R in various forms, either naturally occurring or produced through known techniques. Such forms of TRAIL-R include, but are not limited to, fragments, derivatives, and oligomers of TRAIL-R, as well as fusion proteins containing TRAIL-R or fragments thereof.

TRAIL-R may be modified to create derivatives thereof by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of TRAIL-R may be prepared by linking the chemical moieties to functional groups on TRAIL-R amino acid side chains or at the N-terminus or C-terminus of a TRAIL-R polypeptide. Conjugates comprising diagnostic (detectable) or therapeutic agents attached to TRAIL-R are contemplated herein, as discussed in more detail below.

Other derivatives of TRAIL-R within the scope of this invention include covalent or aggregative conjugates of TRAIL-R polypeptides with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. Examples of fusion proteins are discussed below in connection with TRAIL-

R oligomers. Further, TRAIL-R-containing fusion proteins can comprise peptides added to facilitate purification and identification of TRAIL-R. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., *Bio/Technology* 6:1204, 1988. One such peptide is the Flag® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, which is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein. A murine hybridoma designated 4E11 produces a monoclonal antibody that binds the Flag® peptide in the presence of certain divalent metal cations, as described in U.S. Patent 5,011,912, hereby incorporated by reference. The 4E11 hybridoma cell line has been deposited with the American Type Culture Collection under accession no. HB 9259. Monoclonal antibodies that bind the Flag® peptide are available from Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Connecticut.

One embodiment of the invention is directed to TRAIL-R fragments that retain the ability to bind TRAIL. Such fragments may be prepared by any of a number of conventional techniques. Desired peptide fragments may be chemically synthesized. An alternative involves generating TRAIL-R fragments by enzymatic digestion, e.g., by treating the protein with an enzyme known to cleave proteins at sites defined by particular amino acid residues.

Both cell membrane-bound and soluble (secreted) forms of TRAIL-R are provided herein. Soluble TRAIL-R may be identified (and distinguished from non-soluble membrane-bound counterparts) by separating intact cells expressing a TRAIL-R polypeptide from the culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired protein. The presence of TRAIL-R in the medium indicates that the protein was secreted from the cells and thus is a soluble form of the desired protein.

Soluble forms of receptor proteins typically lack the transmembrane region that would cause retention of the protein on the cell surface. In one embodiment of the invention, a soluble TRAIL-R polypeptide comprises the extracellular domain of the protein. One example of a soluble TRAIL-R is a soluble human TRAIL-R comprising amino acids 52 to 210 of Figure 3.

Soluble forms of TRAIL-R possess certain advantages over the membrane-bound form of the protein. Purification of the protein from recombinant host cells is facilitated, since the soluble proteins are secreted from the cells. Further, soluble proteins are generally more suitable for certain applications, e.g., for intravenous administration.

Naturally occurring variants of the TRAIL-R protein isolated in example 1 are provided herein. Such variants include, for example, proteins that result from alternate mRNA splicing events or from proteolytic cleavage of the TRAIL-R protein. Alternate splicing of mRNA may, for example, yield a truncated but biologically active TRAIL-R protein, such as a naturally occurring soluble form of the protein. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the TRAIL-R protein (generally from 1-5 terminal amino acids). TRAIL-R proteins in which differences in amino acid sequence are attributable to genetic polymorphism (allelic variation among individuals producing the protein) are also contemplated herein.

The skilled artisan will also recognize that the position(s) at which the signal peptide is cleaved may differ from that predicted by computer program, and may vary according to such factors as the type of host cells employed in expressing a recombinant TRAIL-R polypeptide. A preparation of TRAIL-R may include multiple polypeptide species, resulting from cleavage of the signal peptide at different positions.

Isolation of a human TRAIL-R protein is described in example 1. TRAIL-R proteins derived from other mammalian species are contemplated, as well. Probes derived from the DNA sequence of Figure 1 or 2 may be used to screen cDNA libraries derived from other mammalian species, using conventional cross-species hybridization techniques.

Example 1 presents one method for purifying a TRAIL-R protein. Jurkat cells are disrupted, and the subsequent purification process includes affinity chromatography (employing a chromatography matrix containing TRAIL), and reversed phase HPLC.

TRAIL-R polypeptides of the present invention may be purified by any suitable alternative procedure, using known protein purification techniques. In one alternative procedure, the chromatography matrix instead comprises an antibody that binds TRAIL-R. Other cell types expressing TRAIL-R (e.g., the PS-1 cells described in example 2) can be substituted for the Jurkat cells. The cells can be disrupted by any of the numerous known techniques, including freeze-thaw cycling, sonication, mechanical disruption, or by use of cell lysing agents.

The desired degree of purity depends on the intended use of the protein. A relatively high degree of purity is desired when the protein is to be administered *in vivo*, for example. Advantageously, TRAIL-R polypeptides are purified such that no protein bands corresponding to other (non-TRAIL-R) proteins are detectable upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It will be

recognized by one skilled in the pertinent field that multiple bands corresponding to TRAIL-R protein may be visualized by SDS-PAGE, due to differential glycosylation, differential post-translational processing, and the like. TRAIL-R most preferably is purified to substantial homogeneity, as indicated by a single protein band upon analysis by SDS-PAGE. The protein band may be visualized by silver staining, Coomassie blue staining, or (if the protein is radiolabeled) by autoradiography.

The present invention also provides recombinant cloning and expression vectors containing TRAIL-R DNA, as well as host cell containing the recombinant vectors. Expression vectors comprising TRAIL-R DNA may be used to prepare TRAIL-R polypeptides encoded by the DNA. A method for producing TRAIL-R polypeptides comprises culturing host cells transformed with a recombinant expression vector encoding TRAIL-R, under conditions that promote expression of TRAIL-R, then recovering the expressed TRAIL-R polypeptides from the culture. The skilled artisan will recognize that the procedure for purifying the expressed TRAIL-R will vary according to such factors as the type of host cells employed, and whether the TRAIL-R is membrane-bound or a soluble form that is secreted from the host cell.

Any suitable expression system may be employed. The vectors include a DNA encoding a TRAIL-R polypeptide, operably linked to suitable transcriptional or translational regulatory nucleotide sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the TRAIL-R DNA sequence. Thus, a promoter nucleotide sequence is operably linked to an TRAIL-R DNA sequence if the promoter nucleotide sequence controls the transcription of the TRAIL-R DNA sequence. An origin of replication that confers the ability to replicate in the desired host cells, and a selection gene by which transformants are identified, are generally incorporated into the expression vector.

In addition, a sequence encoding an appropriate signal peptide (native or heterologous) can be incorporated into expression vectors. A DNA sequence for a signal peptide (secretory leader) may be fused in frame to the TRAIL-R sequence so that the TRAIL-R is initially translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells promotes extracellular secretion of the TRAIL-R polypeptide. The signal peptide is cleaved from the TRAIL-R polypeptide upon secretion of TRAIL-R from the cell.

Suitable host cells for expression of TRAIL-R polypeptides include prokaryotes, yeast or higher eukaryotic cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual*, Elsevier, New York, (1985). Cell-free translation systems could also be employed to produce TRAIL-R polypeptides using RNAs derived from DNA constructs disclosed herein.

Prokaryotes include gram negative or gram positive organisms, for example, *E. coli* or *Bacilli*. Suitable prokaryotic host cells for transformation include, for example, *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various other species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. In a prokaryotic host cell, such as *E. coli*, a TRAIL-R polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant TRAIL-R polypeptide.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those derived from commercially available plasmids such as the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. An appropriate promoter and a TRAIL DNA sequence are inserted into the pBR322 vector. Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA).

Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include β-lactamase (penicillinase), lactose promoter system (Chang et al., *Nature 275*:615, 1978; and Goeddel et al., *Nature 281*:544, 1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res. 8*:4057, 1980; and EP-A-36776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell expression system employs a phage $\lambda$ $P_L$ promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the $\lambda$ $P_L$ promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9, ATCC 37092) and pPLc28 (resident in *E. coli* RR1, ATCC 53082).

TRAIL-R alternatively may be expressed in yeast host cells, preferably from the *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* or

*Kluyveromyces*, may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* *255*:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* *7*:149, 1968; and Holland et al., *Biochem.* *17*:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EPA-73,657. Another alternative is the glucose-repressible ADH2 promoter described by Russell et al. (*J. Biol. Chem.* *258*:2674, 1982) and Beier et al. (*Nature* *300*:724, 1982). Shuttle vectors replicable in both yeast and *E. coli* may be constructed by inserting DNA sequences from pBR322 for selection and replication in *E. coli* ($Amp^r$ gene and origin of replication) into the above-described yeast vectors.

The yeast α-factor leader sequence may be employed to direct secretion of the TRAIL polypeptide. The α-factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. *See,* e.g., Kurjan et al., *Cell* *30*:933, 1982 and Bitter et al., *Proc. Natl. Acad. Sci. USA* *81*:5330, 1984. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* *75*:1929, 1978. The Hinnen et al. protocol selects for $Trp^+$ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

Yeast host cells transformed by vectors containing an ADH2 promoter sequence may be grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems could also be employed to express recombinant TRAIL-R polypeptides. Bacculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988). Established cell lines of mammalian origin also may be employed. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman et al., *Cell 23*:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines, and the CVI/EBNA cell line derived from the African green monkey kidney cell line CVI (ATCC CCL 70) as described by McMahan et al. (*EMBO J.* 10: 2821, 1991).

Transcriptional and translational control sequences for mammalian host cell expression vectors may be excised from viral genomes. Commonly used promoter sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication (Fiers et al., *Nature 273*:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the SV40 viral origin of replication site is included.

Expression vectors for use in mammalian host cells can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol. 3*:280, 1983), for example. A useful system for stable high level expression of mammalian cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol.* 23:935, 1986). A high expression vector, PMLSV N1/N4, described by Cosman et al., *Nature 312*:768, 1984 has been deposited as ATCC 39890. Additional mammalian expression vectors are described in EP-A-0367566, and in WO 91/18982. As one alternative, the vector may be derived from a retrovirus.

If desired, the native signal peptide of TRAIL-R may be replaced by a heterologous signal peptide or leader sequence. The choice of signal peptide or leader may depend on factors such as the type of host cells in which the recombinant TRAIL-R is to be produced. To illustrate, examples of heterologous signal peptides that are functional in mammalian host cells include the signal sequence for interleukin-7 (IL-7) described in United States Patent 4,965,195, the signal sequence for interleukin-2

receptor described in Cosman et al., *Nature* 312:768 (1984); the interleukin-4 receptor signal peptide described in EP 367,566; the type I interleukin-1 receptor signal peptide described in U.S. Patent 4,968,607; and the type II interleukin-1 receptor signal peptide described in EP 460,846.

Oligomeric Forms of TRAIL-R

Encompassed by the present invention are oligomers that contain TRAIL-R polypeptides. TRAIL-R oligomers may be in the form of covalently-linked or non-covalently-linked dimers, trimers, or higher oligomers.

One embodiment of the invention is directed to oligomers comprising multiple TRAIL-R polypeptides joined *via* covalent or non-covalent interactions between peptide moieties fused to the TRAIL-R polypeptides. Such peptides may be peptide linkers (spacers), or peptides that have the property of promoting oligomerization. Leucine zippers and certain polypeptides derived from antibodies are among the peptides that can promote oligomerization of TRAIL-R polypeptides attached thereto, as described in more detail below.

In particular embodiments, the oligomers comprise from two to four TRAIL-R polypeptides. The TRAIL-R moieties of the oligomer may be soluble polypeptides, as described above.

As one alternative, a TRAIL-R oligomer is prepared using polypeptides derived from immunoglobulins. Preparation of fusion proteins comprising certain heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi et al. (*PNAS USA* 88:10535, 1991); Byrn et al. (*Nature* 344:677, 1990); and Hollenbaugh and Aruffo ("Construction of Immunoglobulin Fusion Proteins", in *Current Protocols in Immunology*, Suppl. 4, pages 10.19.1 - 10.19.11, 1992).

One embodiment of the present invention is directed to a TRAIL-R dimer comprising two fusion proteins created by fusing TRAIL-R to the Fc region of an antibody. A gene fusion encoding the TRAIL-R/Fc fusion protein is inserted into an appropriate expression vector. TRAIL-R/Fc fusion proteins are expressed in host cells transformed with the recombinant expression vector, and allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between the Fc moieties to yield divalent TRAIL-R.

Provided herein are fusion proteins comprising a TRAIL-R polypeptide fused to an Fc polypeptide derived from an antibody. DNA encoding such fusion proteins, as well as dimers containing two fusion proteins joined *via* disulfide bonds between the Fc

moieties thereof, are also provided. The term "Fc polypeptide" as used herein includes native and mutein forms of polypeptides derived from the Fc region of an antibody. Truncated forms of such polypeptides containing the hinge region that promotes dimerization are also included. One suitable Fc polypeptide, described in PCT application WO 93/10151 (hereby incorporated by reference), is a single chain polypeptide extending from the N-terminal hinge region to the native C-terminus of the Fc region of a human IgG1 antibody. Another useful Fc polypeptide is the Fc mutein described in U.S. Patent 5,457,035 and in Baum et al., (*EMBO J.* 13:3992-4001, 1994). The amino acid sequence of this mutein is identical to that of the native Fc sequence presented in WO 93/10151, except that amino acid 19 has been changed from Leu to Ala, amino acid 20 has been changed from Leu to Glu, and amino acid 22 has been changed from Gly to Ala. The mutein exhibits reduced affinity for Fc receptors.

In other embodiments, TRAIL-R may be substituted for the variable portion of an antibody heavy or light chain. If fusion proteins are made with both heavy and light chains of an antibody, it is possible to form a TRAIL-R oligomer with as many as four TRAIL-R extracellular regions.

Alternatively, the oligomer is a fusion protein comprising multiple TRAIL-R polypeptides, with or without peptide linkers (spacer peptides). Among the suitable peptide linkers are those described in U.S. Patents 4,751,180 and 4,935,233, which are hereby incorporated by reference. A DNA sequence encoding a desired peptide linker may be inserted between, and in the same reading frame as, the DNA sequences encoding TRAIL-R, using any suitable conventional technique. For example, a chemically synthesized oligonucleotide encoding the linker may be ligated between sequences encoding TRAIL-R. In particular embodiments, a fusion protein comprises from two to four soluble TRAIL-R polypeptides, separated by peptide linkers.

Another method for preparing oligomeric TRAIL-R involves use of a leucine zipper. Leucine zipper domains are peptides that promote oligomerization of the proteins in which they are found. Leucine zippers were originally identified in several DNA-binding proteins (Landschulz et al., *Science* 240:1759, 1988), and have since been found in a variety of different proteins. Among the known leucine zippers are naturally occurring peptides and derivatives thereof that dimerize or trimerize. Examples of leucine zipper domains suitable for producing soluble oligomeric proteins are described in PCT application WO 94/10308, the leucine zipper derived from lung surfactant protein D (SPD) described in Hoppe et al. (*FEBS Letters* 344:191, 1994) and U.S. Patent application serial no. 08/446,922, hereby incorporated by reference. Recombinant fusion proteins comprising a soluble TRAIL-R polypeptide fused to a

leucine zipper peptide are expressed in suitable host cells, and the soluble oligomeric TRAIL-R that forms is recovered from the culture supernatant.

Oligomeric TRAIL-R has the property of bivalent, trivalent, etc. binding sites for TRAIL. The above-described fusion proteins comprising Fc moieties (and oligomers formed therefrom) offer the advantage of facile purification by affinity chromatography over Protein A or Protein G columns.

Assays

TRAIL-R proteins (including fragments, variants, oligomers, and other forms of TRAIL-R) may be tested for the ability to bind TRAIL in any suitable assay, such as a conventional binding assay. To illustrate, TRAIL-R may be labeled with a detectable reagent (e.g., a radionuclide, chromophore, enzyme that catalyzes a colorimetric or fluorometric reaction, and the like). The labeled TRAIL-R is contacted with cells expressing TRAIL. The cells then are washed to remove unbound labeled TRAIL-R, and the presence of cell-bound label is determined by a suitable technique, chosen according to the nature of the label.

One example of a binding assay procedure is as follows. A recombinant expression vector containing TRAIL cDNA is constructed, e.g., as described in in PCT application WO 97/01633, hereby incorporated by reference. DNA and amino acid sequence information for human and mouse TRAIL is presented in WO 97/01633. TRAIL comprises an N-terminal cytoplasmic domain, a transmembrane region, and a C-terminal extracellular domain. CV1-EBNA-1 cells in 10 $cm^2$ dishes are transfected with the recombinant expression vector. CV-1/EBNA-1 cells (ATCC CRL 10478) constitutively express EBV nuclear antigen-1 driven from the CMV immediate-early enhancer/promoter. CV1-EBNA-1 was derived from the African Green Monkey kidney cell line CV-1 (ATCC CCL 70), as described by McMahan et al. (*EMBO J.* 10:2821, 1991).

The transfected cells are cultured for 24 hours, and the cells in each dish then are split into a 24-well plate. After culturing an additional 48 hours, the transfected cells (about $4 \times 10^4$ cells/well) are washed with BM-NFDM, which is binding medium (RPMI 1640 containing 25 mg/ml bovine serum albumin, 2 mg/ml sodium azide, 20 mM Hepes pH 7.2) to which 50 mg/ml nonfat dry milk has been added. The cells then are incubated for 1 hour at 37°C with various concentrations of a soluble TRAIL-R/Fc fusion protein. Cells then are washed and incubated with a constant saturating concentration of a $^{125}$I-mouse anti-human IgG in binding medium, with gentle agitation for 1 hour at 37°C. After extensive washing, cells are released *via* trypsinization.

The mouse anti-human IgG employed above is directed against the Fc region of human IgG and can be obtained from Jackson Immunoresearch Laboratories, Inc., West Grove, PA. The antibody is radioiodinated using the standard chloramine-T method. The antibody will bind to the Fc portion of any TRAIL-R/Fc protein that has bound to the cells. In all assays, non-specific binding of $^{125}$I-antibody is assayed in the absence of TRAIL-R/Fc, as well as in the presence of TRAIL-R/Fc and a 200-fold molar excess of unlabeled mouse anti-human IgG antibody.

Cell-bound $^{125}$I-antibody is quantified on a Packard Autogamma counter. Affinity calculations (Scatchard, *Ann. N.Y. Acad. Sci.* 51:660, 1949) are generated on RS/1 (BBN Software, Boston, MA) run on a Microvax computer.

Another type of suitable binding assay is a competitive binding assay. To illustrate, biological activity of a TRAIL-R variant may be determined by assaying for the variant's ability to compete with a native TRAIL-R for binding to TRAIL.

Competitive binding assays can be performed by conventional methodology. Reagents that may be employed in competitive binding assays include radiolabeled TRAIL-R and intact cells expressing TRAIL (endogenous or recombinant) on the cell surface. For example, a radiolabeled soluble TRAIL-R fragment can be used to compete with a soluble TRAIL-R variant for binding to cell surface TRAIL. Instead of intact cells, one could substitute a soluble TRAIL/Fc fusion protein bound to a solid phase through the interaction of Protein A or Protein G (on the solid phase) with the Fc moiety. Chromatography columns that contain Protein A and Protein G include those available from Pharmacia Biotech, Inc., Piscataway, NJ. Another type of competitive binding assay utilizes radiolabeled soluble TRAIL, such as a soluble TRAIL/Fc fusion protein, and intact cells expressing TRAIL-R. Qualitative results can be obtained by competitive autoradiographic plate binding assays, while Scatchard plots (Scatchard, *Ann. N.Y. Acad. Sci.* 51:660, 1949) may be utilized to generate quantitative results.

Another type of assay for biological activity involves testing a TRAIL-R polypeptide for the ability to block TRAIL-mediated apoptosis of target cells, such as the human leukemic T-cell line known as Jurkat cells, for example. TRAIL-mediated apoptosis of the cell line designated Jurkat clone E6-1 (ATCC TIB 152) is demonstrated in assay procedures described in PCT application WO 97/01633, hereby incorporated by reference.

<u>Uses of TRAIL-R</u>

Uses of TRAIL-R include, but are not limited to, the following. Certain of these uses of TRAIL-R flow from its ability to bind TRAIL.

TRAIL-R finds use as a protein purification reagent. TRAIL-R polypeptides may be attached to a solid support material and used to purify TRAIL proteins by affinity chromatography. In particular embodiments, a TRAIL-R polypeptide (in any form described herein that is capable of binding TRAIL) is attached to a solid support by conventional procedures. As one example, chromatography columns containing functional groups that will react with functional groups on amino acid side chains of proteins are available (Pharmacia Biotech, Inc., Piscataway, NJ). In an alternative, a TRAIL-R/Fc protein is attached to Protein A- or Protein G-containing chromatography columns through interaction with the Fc moiety.

TRAIL-R proteins also find use in measuring the biological activity of TRAIL proteins in terms of their binding affinity for TRAIL-R. TRAIL-R proteins thus may be employed by those conducting "quality assurance" studies, e.g., to monitor shelf life and stability of TRAIL protein under different conditions. To illustrate, TRAIL-R may be employed in a binding affinity study to measure the biological activity of a TRAIL protein that has been stored at different temperatures, or produced in different cell types. TRAIL-R also may be used to determine whether biological activity is retained after modification of a TRAIL protein (e.g., chemical modification, truncation, mutation, etc.). The binding affinity of the modified TRAIL protein for TRAIL-R is compared to that of an unmodified TRAIL protein to detect any adverse impact of the modifications on biological activity of TRAIL. The biological activity of a TRAIL protein thus can be ascertained before it is used in a research study, for example.

TRAIL-R also finds use in purifying or identifying cells that express TRAIL on the cell surface. TRAIL-R polypeptides are bound to a solid phase such as a column chromatography matrix or a similar suitable substrate. For example, magnetic microspheres can be coated with TRAIL-R and held in an incubation vessel through a magnetic field. Suspensions of cell mixtures containing TRAIL-expressing cells are contacted with the solid phase having TRAIL-R thereon. Cells expressing TRAIL on the cell surface bind to the fixed TRAIL-R, and unbound cells then are washed away.

Alternatively, TRAIL-R can be conjugated to a detectable moiety, then incubated with cells to be tested for TRAIL expression. After incubation, unbound labeled TRAIL-R is removed and the presence or absence of the detectable moiety on the cells is determined.

In a further alternative, mixtures of cells suspected of containing TRAIL$^+$ cells are incubated with biotinylated TRAIL-R. Incubation periods are typically at least one hour in duration to ensure sufficient binding. The resulting mixture then is passed through a column packed with avidin-coated beads, whereby the high affinity of biotin

for avidin provides binding of the desired cells to the beads. Procedures for using avidin-coated beads are known (see Berenson, et al. *J. Cell. Biochem.*, 10D:239, 1986). Washing to remove unbound material, and the release of the bound cells, are performed using conventional methods.

TRAIL-R polypeptides also find use as carriers for delivering agents attached thereto to cells bearing TRAIL. Cells expressing TRAIL include those identified in Wiley et al. (*Immunity*, 3:673-682, 1995). TRAIL-R proteins thus can be used to deliver diagnostic or therapeutic agents to such cells (or to other cell types found to express TRAIL on the cell surface) in *in vitro* or *in vivo* procedures.

Detectable (diagnostic) and therapeutic agents that may be attached to a TRAIL-R polypeptide include, but are not limited to, toxins, other cytotoxic agents, drugs, radionuclides, chromophores, enzymes that catalyze a colorimetric or fluorometric reaction, and the like, with the particular agent being chosen according to the intended application. Among the toxins are ricin, abrin, diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A, ribosomal inactivating proteins, mycotoxins such as trichothecenes, and derivatives and fragments (e.g., single chains) thereof. Radionuclides suitable for diagnostic use include, but are not limited to, $^{123}I$, $^{131}I$, $^{99m}Tc$, $^{111}In$, and $^{76}Br$. Examples of radionuclides suitable for therapeutic use are $^{131}I$, $^{211}At$, $^{77}Br$, $^{186}Re$, $^{188}Re$, $^{212}Pb$, $^{212}Bi$, $^{109}Pd$, $^{64}Cu$, and $^{67}Cu$.

Such agents may be attached to the TRAIL-R by any suitable conventional procedure. TRAIL-R, being a protein, comprises functional groups on amino acid side chains that can be reacted with functional groups on a desired agent to form covalent bonds, for example. Alternatively, the protein or agent may be derivatized to generate or attach a desired reactive functional group. The derivatization may involve attachment of one of the bifunctional coupling reagents available for attaching various molecules to proteins (Pierce Chemical Company, Rockford, Illinois). A number of techniques for radiolabeling proteins are known. Radionuclide metals may be attached to TRAIL-R by using a suitable bifunctional chelating agent, for example.

Conjugates comprising TRAIL-R and a suitable diagnostic or therapeutic agent (preferably covalently linked) are thus prepared. The conjugates are administered or otherwise employed in an amount appropriate for the particular application.

Another use of the protein of the present invention is as a research tool for studying the biological effects that result from inhibiting TRAIL/TRAIL-R interactions on different cell types. TRAIL-R polypeptides also may be employed in *in vitro* assays for detecting TRAIL or TRAIL-R or the interactions thereof.

TRAIL-R may be employed in inhibiting a biological activity of TRAIL, in *in vitro* or *in vivo* procedures. A purified TRAIL-R polypeptide may be used to inhibit binding of TRAIL to endogenous cell surface TRAIL-R. Biological effects that result from the binding of TRAIL to endogenous receptors thus are inhibited. Various forms of TRAIL-R may be employed, including, for example, the above-described TRAIL-R fragments, oligomers, derivatives, and variants that are capable of binding TRAIL. In one embodiment, a soluble TRAIL-R is employed to inhibit a biological activity of TRAIL, e.g., to inhibit TRAIL-mediated apoptosis of particular cells.

TRAIL-R may be administered to a mammal to treat a TRAIL-mediated disorder. Such TRAIL-mediated disorders include conditions caused (directly or indirectly) or exacerbated by TRAIL.

TRAIL-R may be useful for treating thrombotic microangiopathies. One such disorder is thrombotic thrombocytopenic purpura (TTP) (Kwaan, H.C., *Semin. Hematol.*, 24:71, 1987; Thompson et al., *Blood*, 80:1890, 1992). Increasing TTP-associated mortality rates have been reported by the U.S. Centers for Disease Control (Torok et al., *Am. J. Hematol.* 50:84, 1995).

Plasma from patients afflicted with TTP (including $HIV^+$ and $HIV^-$ patients) induces apoptosis of human endothelial cells of dermal microvascular origin, but not large vessel origin (Laurence et al., *Blood*, 87:3245, April 15, 1996). Plasma of TTP patients thus is thought to contain one or more factors that directly or indirectly induce apoptosis. As described in PCT application WO 97/01633 (hereby incorporated by reference), TRAIL is present in the serum of TTP patients, and may play a role in inducing apoptosis of microvascular endothelial cells.

Another thrombotic microangiopathy is hemolytic-uremic syndrome (HUS) (Moake, J.L., *Lancet*, 343:393, 1994; Melnyk et al., (*Arch. Intern. Med.*, 155:2077, 1995; Thompson et al., *supra*). One embodiment of the invention is directed to use of TRAIL-R to treat the condition that is often referred to as "adult HUS" (even though it can strike children as well). A disorder known as childhood/diarrhea-associated HUS differs in etiology from adult HUS.

Other conditions characterized by clotting of small blood vessels may be treated using TRAIL-R. Such conditions include but are not limited to the following. Cardiac problems seen in about 5-10% of pediatric AIDS patients are believed to involve clotting of small blood vessels. Breakdown of the microvasculature in the heart has been reported in multiple sclerosis patients. As a further example, treatment of systemic lupus erythematosus (SLE) is contemplated.

In one embodiment, a patient's blood or plasma is contacted with TRAIL-R *ex vivo*. The TRAIL-R may be bound to a suitable chromatography matrix by conventional procedures. The patient's blood or plasma flows through a chromatography column containing TRAIL-R bound to the matrix, before being returned to the patient. The immobilized receptor binds TRAIL, thus removing TRAIL protein from the patient's blood.

Alternatively, TRAIL-R may be administered *in vivo* to a patient afflicted with a thrombotic microangiopathy. In one embodiment, a soluble form of TRAIL-R is administered to the patient.

The present invention thus provides a method for treating a thrombotic microangiopathy, involving use of an effective amount of TRAIL-R. A TRAIL-R polypeptide may be employed in *in vivo* or *ex vivo* procedures, to inhibit TRAIL-mediated damage to (e.g., apoptosis of) microvascular endothelial cells.

TRAIL-R may be employed in conjunction with other agents useful in treating a particular disorder. In an *in vitro* study reported by Laurence et al. (*Blood* 87:3245, 1996), some reduction of TTP plasma-mediated apoptosis of microvascular endothelial cells was achieved by using an anti-Fas blocking antibody, aurintricarboxylic acid, or normal plasma depleted of cryoprecipitate.

Thus, a patient may be treated with an agent that inhibits Fas-ligand-mediated apoptosis of endothelial cells, in combination with an agent that inhibits TRAIL-mediated apoptosis of endothelial cells. In one embodiment, TRAIL-R and an anti-FAS blocking antibody are both administered to a patient afflicted with a disorder characterized by thrombotic microangiopathy, such as TTP or HUS. Examples of blocking monoclonal antibodies directed against Fas antigen (CD95) are described in PCT application publication number WO 95/10540, hereby incorporated by reference.

TRAIL-R of the present invention may be used in developing treatments for any disorder mediated (directly or indirectly) by defective, or insufficient amounts of, TRAIL-R. TRAIL-R polypeptides may be administered to a mammal afflicted with such a disorder.

Compositions comprising an effective amount of a TRAIL-R polypeptide of the present invention, in combination with other components such as a physiologically acceptable diluent, carrier, or excipient, are provided herein. TRAIL-R can be formulated according to known methods used to prepare pharmaceutically useful compositions. TRAIL-R can be combined in admixture, either as the sole active material or with other known active materials suitable for a given indication, with pharmaceutically acceptable diluents (e.g., saline, Tris-HCl, acetate, and phosphate

buffered solutions), preservatives (e.g., thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable formulations for pharmaceutical compositions include those described in *Remington's Pharmaceutical Sciences*, 16th ed. 1980, Mack Publishing Company, Easton, PA.

In addition, such compositions can contain TRAIL-R complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of TRAIL-R, and are thus chosen according to the intended application. TRAIL-R expressed on the surface of a cell may find use, as well.

Compositions of the present invention may contain a TRAIL-R polypeptide in any form described herein, such as native proteins, variants, derivatives, oligomers, and biologically active fragments. In particular embodiments, the composition comprises a soluble TRAIL-R polypeptide or an oligomer comprising soluble TRAIL-R polypeptides.

TRAIL-R can be administered in any suitable manner, e.g., topically, parenterally, or by inhalation. The term "parenteral" includes injection, e.g., by subcutaneous, intravenous, or intramuscular routes, also including localized administration, e.g., at a site of disease or injury. Sustained release from implants is also contemplated. One skilled in the pertinent art will recognize that suitable dosages will vary, depending upon such factors as the nature of the disorder to be treated, the patient's body weight, age, and general condition, and the route of administration. Preliminary doses can be determined according to animal tests, and the scaling of dosages for human administration are performed according to art-accepted practices.

Antibodies

Antibodies that are immunoreactive with TRAIL-R polypeptides are provided herein. Such antibodies specifically bind TRAIL-R, in that the antibodies bind to TRAIL-R *via* the antigen-binding sites of the antibody (as opposed to non-specific binding).

The TRAIL-R protein prepared as described in example 1 may be employed as an immunogen in producing antibodies immunoreactive therewith. Alternatively, another form of TRAIL-R, such as a fragment or fusion protein, is employed as the immunogen.

Polyclonal and monoclonal antibodies may be prepared by conventional techniques. See, for example, *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Kennet et al. (eds.), Plenum Press, New York (1980); and *Antibodies: A Laboratory Manual*, Harlow and Land (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1988). Production of monoclonal antibodies directed against TRAIL-R is further illustrated in example 4.

Antigen-binding fragments of such antibodies, which may be produced by conventional techniques, are also encompassed by the present invention. Examples of such fragments include, but are not limited to, Fab and $F(ab')_2$ fragments. Antibody fragments and derivatives produced by genetic engineering techniques are also provided.

The monoclonal antibodies of the present invention include chimeric antibodies, e.g., humanized versions of murine monoclonal antibodies. Such humanized antibodies may be prepared by known techniques, and offer the advantage of reduced immunogenicity when the antibodies are administered to humans. In one embodiment, a humanized monoclonal antibody comprises the variable region of a murine antibody (or just the antigen binding site thereof) and a constant region derived from a human antibody. Alternatively, a humanized antibody fragment may comprise the antigen binding site of a murine monoclonal antibody and a variable region fragment (lacking the antigen-binding site) derived from a human antibody. Procedures for the production of chimeric and further engineered monoclonal antibodies include those described in Riechmann et al. (*Nature 332*:323, 1988), Liu et al. (*PNAS 84*:3439, 1987), Larrick et al. (*Bio/Technology 7*:934, 1989), and Winter and Harris (*TIPS 14*:139, May, 1993).

Among the uses of the antibodies is use in assays to detect the presence of TRAIL-R polypeptides, either *in vitro* or *in vivo*. The antibodies also may be employed in purifying TRAIL-R proteins by immunoaffinity chromatography.

Those antibodies that additionally can block binding of TRAIL-R to TRAIL may be used to inhibit a biological activity that results from such binding. Such blocking antibodies may be identified using any suitable assay procedure, such as by testing antibodies for the ability to inhibit binding of TRAIL to cells expressing TRAIL-R. Examples of such cells are the Jurkat cells and PS1 cells described in example 2 below. Alternatively, blocking antibodies may be identified in assays for the ability to inhibit a biological effect that results from binding of TRAIL to target cells. Antibodies may be assayed for the ability to inhibit TRAIL-mediated lysis of Jurkat cells, for example.

Such an antibody may be employed in an *in vitro* procedure, or administered *in vivo* to inhibit a TRAIL-R-mediated biological activity. Disorders caused or exacerbated (directly or indirectly) by the interaction of TRAIL with cell surface TRAIL receptor thus may be treated. A therapeutic method involves *in vivo* administration of a blocking antibody to a mammal in an amount effective in inhibiting a TRAIL-mediated biological activity. Disorders caused or exacerbated by TRAIL, directly or indirectly, are thus treated. Monoclonal antibodies are generally preferred for use in such therapeutic methods. In one embodiment, an antigen-binding antibody fragment is employed.

A blocking antibody directed against TRAIL-R may be substituted for TRAIL-R in the above-described method of treating thrombotic microangiopathy, e.g., in treating TTP or HUS. The antibody is administered *in vivo*, to inhibit TRAIL-mediated damage to (e.g., apoptosis of) microvascular endothelial cells.

Compositions comprising an antibody that is directed against TRAIL-R, and a physiologically acceptable diluent, excipient, or carrier, are provided herein. Suitable components of such compositions are as described above for compositions containing TRAIL-R proteins.

Also provided herein are conjugates comprising a detectable (e.g., diagnostic) or therapeutic agent, attached to an antibody directed against TRAIL-R. Examples of such agents are presented above. The conjugates find use in *in vitro* or *in vivo* procedures.

Nucleic Acids and Uses Thereof

The present invention provides TRAIL-R nucleic acids. Such nucleic acids include, but are not limited to, DNA encoding the peptides described in example 2. Such DNAs can be identified from knowledge of the genetic code. One embodiment of the invention is directed to fragments of TRAIL-R nucleotide sequences comprising at least about 17 contiguous nucleotides of a TRAIL-R DNA sequence. Nucleic acids provided herein include DNA and RNA complements of said fragments, along with both single-stranded and double-stranded forms of the TRAIL-R DNA.

Among the uses of TRAIL-R nucleic acid fragments is use as probes or primers. Using knowledge of the genetic code in combination with the amino acid sequences set forth in example 2, sets of degenerate oligonucleotides can be prepared. Such oligonucleotides find use as primers, e.g., in polymerase chain reactions (PCR), whereby TRAIL-R DNA fragments are isolated and amplified.

The following examples are provided to further illustrate particular embodiments of the invention, and are not to be construed as limiting the scope of the present invention.

## EXAMPLE 1: Purification of TRAIL-R Protein

A human TRAIL receptor (TRAIL-R) protein was prepared by the following procedure. Trail-R was isolated from the cell membranes of Jurkat cells, a human acute T leukemia cell line. Jurkat cells were chosen because a specific band can be affinity precipitated from surface-biotinylated Jurkat cells, using Flag®-TRAIL covalently coupled to affi-gel beads (Biorad Laboratories, Richmond, CA). The precipitated band has a molecular weight of about 52 kD. A minor specific band of about 42 kD also was present, possibly accounting for a proteolytic breakdown product or a less glycosylated form of TRAIL-R.

Approximately 50 billion Jurkat cells were harvested by centrifugation (80 ml of cell pellet), washed once with PBS, then shock frozen on liquid nitrogen. Plasma membranes were isolated according to method number three described in Maeda et al., *Biochim. et Biophys. Acta*, 731:115, 1983; hereby incorporated by reference) with five modifications:

1. The following protease inhibitors were included in all solutions at the indicated concentrations: Aprotinin, 150 nM; EDTA, 5 mM; Leupeptin, 1 μM; pA-PMSF, 20 μM; Pefabloc, 500 μM; Pepstatin A, 1 μM; PMSF, 500 μM.
2. Dithiothreitol was not used.
3. DNAase was not used in the homogenization solution.
4. 1.25 ml of homogenization buffer was used per ml of cell pellet.
5. The homogenization was accomplished by five passages through a ground glass dounce homogenizer.

After isolation of the cell membranes, proteins were solubilized by resuspending the isolated membranes in 50 ml PBS containing 1% octylglucoside and all of the above mentioned protease inhibitors at the above indicated concentrations. The resulting solution was then repeatedly vortexed during a thirty-minute incubation at 4°C. The solution was then centrifuged at 20,000 rpm in an SW28 rotor in an LE-80 Beckman ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) at 4°C for 30 minutes to obtain the supernatant (the membrane extract).

Chromatography

The first step of purification of TRAIL-R out of the membrane extract prepared above was affinity chromatography. The membrane extract was first applied to an anti-Flag® M2 affi-gel column (10 mg of monoclonal antibody M2 coupled to 2 ml of Affi-gel beads) to adsorb any nonspecifically binding material. The flow-through was then applied to a Flag®-TRAIL affi-gel column (10 mg of recombinant protein coupled to 1 ml of affi-gel beads).

The Affi-gel support is an N-hydroxysuccinimide ester of a derivatized, crosslinked agarose gel bead (available from Biorad Laboratories, Richmond, CA). As discussed above, the Flag® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, provides an epitope reversibly bound by specific monoclonal antibodies, enabling rapid assay and facile purification of expressed recombinant protein. M2 is a monoclonal antibody that binds Flag®. Monoclonal antibodies that bind the Flag® peptide, as well as other reagents for preparing and using Flag® fusion proteins, are available from Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Connecticut. Preparation of Flag®-TRAIL fusion proteins (comprising Flag® fused to a soluble TRAIL polypeptide) is further described in PCT application WO 97/01633, hereby incorporated by reference.

The column was washed with 25 ml of each of the following buffers, in the order indicated:

1. PBS containing 1% octylglucoside
2. PBS
3. PBS containing an additional 200 mM NaCl
4. PBS

The bound material was eluted with 50 mM Na Citrate (pH 3) in 1 ml fractions and immediately neutralized with 300 µl of 1 M Tris-HCl (pH 8.5) per fraction. The TRAIL-binding activity of each fraction was determined by a TRAIL-R-specific ELISA as described below. Fractions with high TRAIL-binding activity were pooled, brought to 0.1 % Trifluoroacetic acid (TFA), and subsequently chromatographed on a capillary reversed-phase HPLC column [500 µm internal diameter X 25 cm fused silicone capillary column packed with 5 µm Vydac $C_4$ material (Vydac, Hesperia, CA)] using a linear gradient (2% per minute) from 0% to 100% acetonitrile in water containing 0.1% TFA. Fractions containing high TRAIL-binding activity are then determined as above, pooled, and, if desired, lyophylized.

TRAIL-R-specific ELISA:

Serial dilutions of TRAIL-R-containing samples (in 50 mM $NaHCO_3$, brought to pH 9 with NaOH) were coated onto Linbro/Titertek 96 well flat bottom E.I.A. microtitration plates (ICN Biomedicals Inc., Aurora, OH) at 100 µl/well. After incubation at 4°C for 16 hours, the wells were washed six times with 200 µl PBS containing 0.05% Tween-20 (PBS-Tween). The wells were then incubated with Flag®-TRAIL at 1 µg/ml in PBS-Tween with 5% fetal calf serum (FCS) for 90 minutes (100 µl per well), followed by washing as above. Next, each well was incubated with the anti-Flag® monoclonal antibody M2 at 1 µg/ml in PBS-Tween containing 5% FCS for 90 minutes (100 µl per well), followed by washing as above. Subsequently, wells were incubated with a polyclonal goat anti-mIgG1-specific horseradish peroxidase-conjugated antibody (a 1:5000 dilution of the commercial stock in PBS-Tween containing 5% FCS) for 90 minutes (100 µl per well). The HRP-conjugated antibody was obtained from Southern Biotechnology Associates, Inc., Birmingham, Alabama. Wells then were washed six times, as above.

For development of the ELISA, a substrate mix [100 µl per well of a 1:1 premix of the TMB Peroxidase Substrate and Peroxidase Solution B (Kirkegaard Perry Laboratories, Gaithersburg, Maryland)] was added to the wells. After sufficient color reaction, the enzymatic reaction was terminated by addition of 2 N $H_2SO_4$ (50 µl per well). Color intensity (indicating TRAIL-binding activity) was determined by measuring extinction at 450 nm on a V Max plate reader (Molecular Devices, Sunnyvale, CA).

## EXAMPLE 2: Amino Acid Sequence

(a) TRAIL-R purified from Jurkat cells

TRAIL-R protein isolated from Jurkat cells was digested with trypsin, using conventional procedures. Amino acid sequence analysis was conducted on one of the peptide fragments produced by the tryptic digest. The fragment was found to contain the sequence:

VPANEGD

(b) TRAIL-R purified from PS-1 cells

TRAIL-R protein was also isolated from PS-1 cells. PS-1 is a human B cell line that spontaneously arose after lethal irradiation of human peripheral blood

lymphocytes (PBLs). The TRAIL-R protein was digested with trypsin, using conventional procedures. Amino acid sequence analysis was conducted on peptide fragments that resulted from the tryptic digest. One of the fragments was found to contain the sequence:

VPANEGD

Two other fragments were found to contain the following amino acid sequences, respectively:

VCEC
SGEVELSSV

## EXAMPLE 3: DNA and Amino Acid Sequences

The amino acid sequence of additional tryptic digest peptide fragments of TRAIL-R was determined. Degenerate oligonucleotides, based upon the amino acid sequence of two of the peptides, were prepared. A TRAIL-R DNA fragment was isolated and amplified by polymerase chain reaction (PCR), using the degenerate oligonucleotides as 5' and 3' primers. The PCR was conducted according to conventional procedures, using cDNA derived from the PS-1 cell line described in example 2 as the template. The nucleotide sequence of the isolated TRAIL-R DNA fragment, and the amino acid sequence encoded thereby, are presented in Figure 1.

The amino acid sequence in Figure 1 bears significant homology to the so-called death domains found in certain other receptors. The cytoplasmic region of Fas and TNF receptor type I each contain a death domain, which is associated with transduction of an apoptotic signal (Tartaglia et al. *Cell* 74:845, 1993; Itoh and Nagata, *J. Biol. Chem.* 268:10932, 1993). Thus, the sequence presented in Figure 1 is believed to be found within the cytoplasmic domain of TRAIL-R.

A probe derived from the fragment isolated above (the fragment of Figure 1) was used to screen a cDNA library (human foreskin fibroblast-derived cDNA in λgt10 vector), and a human TRAIL-R cDNA was isolated. The nucleotide sequence of the coding region of this cDNA is presented in Figure 2, and the amino acid sequence encoded thereby is shown in Figure 3.

## EXAMPLE 4: Monoclonal Antibodies That Bind TRAIL-R

This example illustrates a method for preparing monoclonal antibodies that bind TRAIL-R. Suitable immunogens that may be employed in generating such antibodies

include, but are not limited to, purified TRAIL-R protein or an immunogenic fragment thereof such as the extracellular domain, or fusion proteins containing TRAIL-R (e.g., a soluble TRAIL-R/Fc fusion protein).

Purified TRAIL-R can be used to generate monoclonal antibodies immunoreactive therewith, using conventional techniques such as those described in U.S. Patent 4,411,993. Briefly, mice are immunized with TRAIL-R immunogen emulsified in complete Freund's adjuvant, and injected in amounts ranging from 10-100 μg subcutaneously or intraperitoneally. Ten to twelve days later, the immunized animals are boosted with additional TRAIL-R emulsified in incomplete Freund's adjuvant. Mice are periodically boosted thereafter on a weekly to bi-weekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision to test for TRAIL-R antibodies by dot blot assay, ELISA (Enzyme-Linked Immunosorbent Assay) or inhibition of TRAIL binding.

Following detection of an appropriate antibody titer, positive animals are provided one last intravenous injection of TRAIL-R in saline. Three to four days later, the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line, e.g., NS1 or preferably P3x63Ag8.653 (ATCC CRL 1580). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells are screened by ELISA for reactivity against purified TRAIL-R by adaptations of the techniques disclosed in Engvall et al., *Immunochem.* 8:871, 1971 and in U.S. Patent 4,703,004. A preferred screening technique is the antibody capture technique described in Beckmann et al., (*J. Immunol.* 144:4212, 1990) Positive hybridoma cells can be injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-TRAIL-R monoclonal antibodies. Alternatively, hybridoma cells can be grown *in vitro* in flasks or roller bottles by various techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to Protein A or Protein G can also be used, as can affinity chromatography based upon binding to TRAIL-R.

What is claimed is:

1. A purified TRAIL receptor (TRAIL-R) polypeptide that is capable of binding TRAIL, wherein the TRAIL-R is characterized as comprising the amino acid sequence VPANEGD.

2. A TRAIL-R polypeptide of claim 1, wherein said polypeptide is further characterized by a molecular weight of about 50 to 55 kilodaltons.

3. A TRAIL-R polypeptide of claim 1, wherein said polypeptide is further characterized by comprising the amino acid sequence ETLRQCFDDFADLVPFDS WEPLMRKLGLMDNEIKVAKAEAAGHRDTLXTML.

4. A TRAIL-R polypeptide of claim 3, wherein said polypeptide is further characterized by a molecular weight of about 50 to 55 kilodaltons.

5. A purified TRAIL-R polypeptide, wherein said polypeptide is a fragment of the TRAIL-R polypeptide of claim 1, wherein said fragment is capable of binding TRAIL.

6. A purified TRAIL-R polypeptide selected from the group consisting of:

a) a polypeptide comprising amino acids 52 to 440 of Figure 3; and

b) a fragment of the polypeptide of (a), wherein said fragment is capable of binding TRAIL.

7. A TRAIL-R polypeptide of claim 6, wherein said polypeptide comprises amino acids 52 to 440 of the sequence presented in Figure 3.

8. A TRAIL-R polypeptide of claim 6, wherein said fragment is a soluble TRAIL-R comprising the extracellular domain of the TRAIL-R protein of Figure 3.

9. An oligomer comprising from two to four TRAIL-R polypeptides of claim 1, 3, 6, or 8.

10. An oligomer of claim 9, wherein said oligomer is a dimer comprising two soluble TRAIL-R/Fc fusion proteins.

11. A composition comprising a TRAIL-R polypeptide of claim 1, 2, 3, 6, 7, or 8, and a physiologically acceptable diluent, excipient, or carrier.

12. A composition comprising an oligomer of claim 9 and a physiologically acceptable diluent, excipient, or carrier.

13. An isolated TRAIL-R DNA, wherein said DNA comprises the nucleotide sequence presented in Figure 1.

14. An isolated TRAIL-R DNA, wherein said DNA encodes a polypeptide selected from the group consisting of:

a) a polypeptide comprising residues 1 to 440 of Figure 3;

b) a polypeptide comprising residues 52 to 440 of Figure 3; and

c) a fragment of the polypeptide of (a) or (b), wherein said fragment is capable of binding TRAIL.

15. A TRAIL-R DNA of claim 14, wherein said DNA encodes a polypeptide selected from the group consisting of:

a) a polypeptide comprising residues 1 to 440 of Figure 3; and

b) a polypeptide comprising residues 52 to 440 of Figure 3.

16. An antibody that is directed against a TRAIL-R polypeptide of claim 1, 3, 6, 7, or 8.

17. An antibody of claim 16, wherein the antibody is a monoclonal antibody.

## ABSTRACT OF THE DISCLOSURE

The invention is directed to a protein designated TRAIL receptor, which binds the protein known as TNF-Related Apoptosis-Inducing Ligand (TRAIL). The TRAIL receptor finds use in purifying TRAIL or inhibiting activities thereof. Antibodies that are immunoreactive with TRAIL-R are also provided.

# FIGURE 1

```
    CTGAGACTCTGAGACAGTGCTTCGATGACTTTGCAGACTTGGTGCCCTTTGACTCCTGGG
  1 ---------+---------+---------+---------+---------+---------+ 60
    GACTCTGAGACTCTGTCACGAAGCTACTGAAACGTCTGAACCACGGGAAACTGAGGACCC

     E  T  L  R  Q  C  F  D  D  F  A  D  L  V  P  F  D  S  W  E -

    AGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGG
 61 ---------+---------+---------+---------+---------+---------+ 120
    TCGGCGAGTACTCCTTCAACCCGGAGTACCTGTTACTCTATTTCCACCGATTTCGACTCC

     P  L  M  R  K  L  G  L  M  D  N  E  I  K  V  A  K  A  E  A -

    CAGCGGGCCACAGGGACACCTTGTNCACNATGCTGAT
121 ---------+---------+---------+------- 157
    GTCGCCCGGTGTCCCTGTGGAACANGTGNTACGACTA

     A  G  H  R  D  T  L  X  T  M  L     -
```

# FIGURE 2

<u>ATG</u>GAACAACGGGGACAGAACGCCCCGGCCGCTTCGGGGGCCCGGAAAAG
GCACGGCCCAGGACCCAGGGAGGCGCGGGGAGCCAGGCCTGGGCCCCGGG
TCCCCAAGACCCTTGTGCTCGTTGTCGCCGCGGTCCTGCTGTTGGTCTCA
GCTGAGTCTGCTCTGATCACCCAACAAGACCTAGCTCCCCAGCAGAGAGC
GGCCCCACAACAAAAGAGGTCCAGCCCCTCAGAGGGATTGTGTCCACCTG
GACACCATATCTCAGAAGACGGTAGAGATTGCATCTCCTGCAAATATGGA
CAGGACTATAGCACTCACTGGAATGACCTCCTTTTCTGCTTGCGCTGCAC
CAGGTGTGATTCAGGTGAAGTGGAGCTAAGTCCGTGCACCACGACCAGAA
ACACAGTGTGTCAGTGCGAAGAAGGCACCTTCCGGGAAGAAGATTCTCCT
GAGATGTGCCGGAAGTGCCGCACAGGGTGTCCCAGAGGGATGGTCAAGGT
CGGTGATTGTACACCCTGGAGTGACATCGAATGTGTCCACAAAGAATCAG
GTACAAAGCACAGTGGGGAAGCCCCAGCTGTGGAGGAGACGGTGACCTCC
AGCCCAGGGACTCCTGCCTCTCCCTGTTCTCTCTCAGGCATCATCATAGG
AGTCACAGTTGCAGCCGTAGTCTTGATTGTGGCTGTGTTTGTTTGCAAGT
CTTTACTGTGGAAGAAAGTCCTTCCTTACCTGAAAGGCATCTGCTCAGGT
GGTGGTGGGGACCCTGAGCGTGTGGACAGAAGCTCACAACGACCTGGGGC
TGAGGACAATGTCCTCAATGAGATCGTGAGTATCTTGCAGCCCACCCAGG
TCCCTGAGCAGGAAATGGAAGTCCAGGAGCCAGCAGAGCCAACAGGTGTC
AACATGTTGTCCCCCGGGGAGTCAGAGCATCTGCTGGAACCGGCAGAAGC
TGAAAGGTCTCAGAGGAGGAGGCTGCTGGTTCCAGCAAATGAAGGTGATC
CCACTGAGACTCTGAGACAGTGCTTCGATGACTTTGCAGACTTGGTGCCC
TTTGACTCCTGGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGA
GATAAAGGTGGCTAAAGCTGAGGCAGCGGGCCACAGGGACACCTTGTACA
CGATGCTGATAAAGTGGGTCAACAAAACCGGGCGAGATGCCTCTGTCCAC
ACCCTGCTGGATGCCTTGGAGACGCTGGGAGAGAGACTTGCCAAGCAGAA
GATTGAGGACCACTTGTTGAGCTCTGGAAAGTTCATGTATCTAGAAGGTA
ATGCAGACTCTGCCATGTCC<u>TAA</u>

# FIGURE 3

| | | | | | |
|---|---|---|---|---|---|
| 1 | MEQRGQNAPA | ASGARKRHGP | GPREARGARP | GPRVPKTLVL | VVAAVLLLVS |
| 51 | AESALITQQD | LAPQQRAAPQ | QKRSSPSEGL | CPPGHHISED | GRDCISCKYG |
| 101 | QDYSTHWNDL | LFCLRCTRCD | SGEVELSPCT | TTRNTVCQCE | EGTFREEDSP |
| 151 | EMCRKCRTGC | PRGMVKVGDC | TPWSDIECVH | KESGTKHSGE | APAVEETVTS |
| 201 | SPGTPASPCS | LSGIIIGVTV | AAVVLIVAVF | VCKSLLWKKV | LPYLKGICSG |
| 251 | GGGDPERVDR | SSQRPGAEDN | VLNEIVSILQ | PTQVPEQEME | VQEPAEPTGV |
| 301 | NMLSPGESEH | LLEPAEAERS | QRRRLLVPAN | EGDPTETLRQ | CFDDFADLVP |
| 351 | FDSWEPLMRK | LGLMDNEIKV | AKAEAAGHRD | TLYTMLIKWV | NKTGRDASVH |
| 401 | TLLDALETLG | ERLAKQKIED | HLLSSGKFMY | LEGNADSAMS | |

# EXHIBIT E

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## TITLE

## RECEPTOR THAT BINDS TRAIL

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application serial no. ___________, filed February 13, 1997, currently pending.

## BACKGROUND OF THE INVENTION

A protein known as TNF-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family of ligands (Wiley et al., *Immunity*, 3:673-682, 1995). TRAIL has demonstrated the ability to induce apoptosis of certain transformed cells, including a number of different types of cancer cells as well as virally infected cells (PCT application WO 97/01633 and Wiley et al., *supra*).

Identification of receptor protein(s) that bind TRAIL would prove useful in further study of the biological activities of TRAIL. However, prior to the present invention, no receptor for TRAIL had been reported.

## SUMMARY OF THE INVENTION

The present invention is directed to a novel protein designated TRAIL receptor (TRAIL-R). TRAIL-R binds to a protein known as TNF-related apoptosis-inducing ligand (TRAIL). Antibodies that are immunoreactive with TRAIL-R are also provided.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 presents the nucleotide sequence of a TRAIL receptor DNA fragment, as well as the amino acid sequence encoded thereby. This DNA fragment was isolated as described in Example 3.

## DETAILED DESCRIPTION OF THE INVENTION

A novel protein designated TRAIL receptor (TRAIL-R) is provided herein. TRAIL-R binds to the cytokine designated TNF-related apoptosis-inducing ligand (TRAIL). Certain uses of TRAIL-R flow from this ability to bind TRAIL, as discussed further below. TRAIL-R finds use in inhibiting biological activities of TRAIL, or in purifying TRAIL by affinity chromatography, for example.

TRAIL-R protein or immunogenic fragments thereof may be employed as immunogens to generate antibodies that are immunoreactive therewith. In one embodiment of the invention, the antibodies are monoclonal antibodies.

A human TRAIL-R protein was purified as described in example 1. In example 2, amino acid sequence information derived from fragments of TRAIL-R is presented. One embodiment of the invention is directed to a purified human TRAIL-R protein that is capable of binding TRAIL, wherein the TRAIL-R is characterized as comprising the amino acid sequences VPANEGD, VCEC, SGEVELSSV, and ETLRQCFDDFADL VPFDSWEPLMRKLGLMDNEIKVAKAEAAGHRDTLXTML. Also provided are TRAIL-R fragments comprising from one to three of the characterizing amino acid sequences.

The nucleotide sequence of a TRAIL-R DNA fragment, and the amino acid sequence encoded thereby, are presented in Figure 1. This DNA fragment was isolated by polymerase chain reaction, as described in example 3. The amino acid sequence presented in Figure 1 has characteristics of the so-called "death domains" found in the cytoplasmic region of certain other receptor proteins. Such domains have been reported to be associated with transduction of apoptotic signals. The cytoplasmic region of Fas and TNF receptor type I each contain a death domain (Tartaglia et al. *Cell* 74:845, 1993; Itoh and Nagata, *J. Biol. Chem.* 268:10932, 1993).

In one embodiment, the protein is further characterized by a molecular weight within the range of about 50 to 55 kilodaltons, which is the molecular weight determined for a preparation of native, full length, human TRAIL-R. Molecular weight can be determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The skilled artisan will recognize that the molecular weight of particular preparations of TRAIL-R protein may differ, according to such factors as the degree of glycosylation. The glycosylation pattern of a particular preparation of TRAIL-R may vary according to the type of cells in which the protein is expressed, for example. Further, a given preparation may include multiple differentially glycosylated species of the protein. TRAIL-R polypeptides with or without associated native-pattern glycosylation are provided herein.

As used herein, the term "TRAIL-R" refers to a genus of polypeptides that are substantially homologous to the human TRAIL-R protein isolated in example 1. The present invention encompasses TRAIL-R in various forms, either naturally occurring or produced through known techniques. Such forms of TRAIL-R include, but are not limited to, fragments, derivatives, and oligomers of TRAIL-R, as well as fusion proteins containing TRAIL-R or fragments thereof.

TRAIL-R may be modified to create derivatives thereof by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of TRAIL-R may be prepared by linking the chemical moieties to functional groups on TRAIL-R amino acid side chains or at the N-terminus or C-terminus of a TRAIL-R polypeptide. Conjugates comprising diagnostic (detectable) or therapeutic agents attached to TRAIL-R are contemplated herein, as discussed in more detail below.

Other derivatives of TRAIL-R within the scope of this invention include covalent or aggregative conjugates of TRAIL-R polypeptides with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. Examples of fusion proteins are discussed below in connection with TRAIL-R oligomers. Further, TRAIL-R-containing fusion proteins can comprise peptides added to facilitate purification and identification of TRAIL-R. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., *Bio/Technology* 6:1204, 1988. One such peptide is the Flag® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, which is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein. A murine hybridoma designated 4E11 produces a monoclonal antibody that binds the Flag® peptide in the presence of certain divalent metal cations, as described in U.S. Patent 5,011,912, hereby incorporated by reference. The 4E11 hybridoma cell line has been deposited with the American Type Culture Collection under accession no. HB 9259. Monoclonal antibodies that bind the Flag® peptide are available from Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Connecticut.

One embodiment of the invention is directed to TRAIL-R fragments that retain the ability to bind TRAIL. Such fragments may be prepared by any of a number of conventional techniques. Desired peptide fragments may be chemically synthesized. An alternative involves generating TRAIL-R fragments by enzymatic digestion, e.g., by treating the protein with an enzyme known to cleave proteins at sites defined by particular amino acid residues.

Both cell membrane-bound and soluble (secreted) forms of TRAIL-R are provided herein. Soluble TRAIL-R may be identified (and distinguished from non-soluble membrane-bound counterparts) by separating intact cells expressing a TRAIL-R polypeptide from the culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired protein. The presence of TRAIL-R in the medium indicates that the protein was secreted from the cells and thus is a soluble form

of the desired protein. Soluble forms of receptor proteins typically lack the transmembrane region that would cause retention of the protein on the cell surface.

Soluble forms of TRAIL-R possess certain advantages over the membrane-bound form of the protein. Purification of the protein from recombinant host cells is facilitated, since the soluble proteins are secreted from the cells. Further, soluble proteins are generally more suitable for certain applications, e.g., for intravenous administration.

Naturally occurring variants of the TRAIL-R protein isolated in example 1 are provided herein. Such variants include, for example, proteins that result from alternate mRNA splicing events or from proteolytic cleavage of the TRAIL-R protein. Alternate splicing of mRNA may, for example, yield a truncated but biologically active TRAIL-R protein, such as a naturally occurring soluble form of the protein. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the TRAIL-R protein (generally from 1-5 terminal amino acids). TRAIL-R proteins in which differences in amino acid sequence are attributable to genetic polymorphism (allelic variation among individuals producing the protein) are also contemplated herein.

Isolation of a human TRAIL-R protein is described in example 1. TRAIL-R proteins derived from other mammalian species are contemplated, as well.

Example 1 presents one method for purifying a TRAIL-R protein. Jurkat cells are disrupted, and the subsequent purification process includes affinity chromatography (employing a chromatography matrix containing TRAIL), and reversed phase HPLC.

TRAIL-R polypeptides of the present invention may be purified by any suitable alternative procedure, using known protein purification techniques. In one alternative procedure, the chromatography matrix instead comprises an antibody that binds TRAIL-R. Other cell types expressing TRAIL-R (e.g., the PS-1 cells described in example 2) can be substituted for the Jurkat cells. The cells can be disrupted by any of the numerous known techniques, including freeze-thaw cycling, sonication, mechanical disruption, or by use of cell lysing agents.

The desired degree of purity depends on the intended use of the protein. A relatively high degree of purity is desired when the protein is to be administered *in vivo*, for example. Advantageously, TRAIL-R polypeptides are purified such that no protein bands corresponding to other (non-TRAIL-R) proteins are detectable upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It will be recognized by one skilled in the pertinent field that multiple bands corresponding to

TRAIL-R protein may be visualized by SDS-PAGE, due to differential glycosylation, differential post-translational processing, and the like. TRAIL-R most preferably is purified to substantial homogeneity, as indicated by a single protein band upon analysis by SDS-PAGE. The protein band may be visualized by silver staining, Coomassie blue staining, or (if the protein is radiolabeled) by autoradiography.

Oligomeric Forms of TRAIL-R

Encompassed by the present invention are oligomers that contain TRAIL-R polypeptides. TRAIL-R oligomers may be in the form of covalently-linked or non-covalently-linked dimers, trimers, or higher oligomers.

One embodiment of the invention is directed to oligomers comprising multiple TRAIL-R polypeptides joined *via* covalent or non-covalent interactions between peptide moieties fused to the TRAIL-R polypeptides. Such peptides may be peptide linkers (spacers), or peptides that have the property of promoting oligomerization. Leucine zippers and certain polypeptides derived from antibodies are among the peptides that can promote oligomerization of TRAIL-R polypeptides attached thereto, as described in more detail below.

In particular embodiments, the oligomers comprise from two to four TRAIL-R polypeptides. The TRAIL-R moieties of the oligomer may be soluble polypeptides, as described above.

As one alternative, a TRAIL-R oligomer is prepared using polypeptides derived from immunoglobulins. Preparation of fusion proteins comprising certain heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi et al. (*PNAS USA* 88:10535, 1991); Byrn et al. (*Nature* 344:677, 1990); and Hollenbaugh and Aruffo ("Construction of Immunoglobulin Fusion Proteins", in *Current Protocols in Immunology*, Suppl. 4, pages 10.19.1 - 10.19.11, 1992).

One embodiment of the present invention is directed to a TRAIL-R dimer comprising two fusion proteins created by fusing TRAIL-R to the Fc region of an antibody. A gene fusion encoding the TRAIL-R/Fc fusion protein is inserted into an appropriate expression vector. TRAIL-R/Fc fusion proteins are expressed in host cells transformed with the recombinant expression vector, and allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between the Fc moieties to yield divalent TRAIL-R.

Provided herein are fusion proteins comprising a TRAIL-R polypeptide fused to an Fc polypeptide derived from an antibody. DNA encoding such fusion proteins, as

well as dimers containing two fusion proteins joined *via* disulfide bonds between the Fc moieties thereof, are also provided. The term "Fc polypeptide" as used herein includes native and mutein forms of polypeptides derived from the Fc region of an antibody. Truncated forms of such polypeptides containing the hinge region that promotes dimerization are also included. One suitable Fc polypeptide, described in PCT application WO 93/10151 (hereby incorporated by reference), is a single chain polypeptide extending from the N-terminal hinge region to the native C-terminus of the Fc region of a human IgG1 antibody. Another useful Fc polypeptide is the Fc mutein described in U.S. Patent 5,457,035 and in Baum et al., (*EMBO J.* 13:3992-4001, 1994). The amino acid sequence of this mutein is identical to that of the native Fc sequence presented in WO 93/10151, except that amino acid 19 has been changed from Leu to Ala, amino acid 20 has been changed from Leu to Glu, and amino acid 22 has been changed from Gly to Ala. The mutein exhibits reduced affinity for Fc receptors.

In other embodiments, TRAIL-R may be substituted for the variable portion of an antibody heavy or light chain. If fusion proteins are made with both heavy and light chains of an antibody, it is possible to form a TRAIL-R oligomer with as many as four TRAIL-R extracellular regions.

Alternatively, the oligomer is a fusion protein comprising multiple TRAIL-R polypeptides, with or without peptide linkers (spacer peptides). Among the suitable peptide linkers are those described in U.S. Patents 4,751,180 and 4,935,233, which are hereby incorporated by reference. A DNA sequence encoding a desired peptide linker may be inserted between, and in the same reading frame as, the DNA sequences encoding TRAIL-R, using any suitable conventional technique. For example, a chemically synthesized oligonucleotide encoding the linker may be ligated between sequences encoding TRAIL-R. In particular embodiments, a fusion protein comprises from two to four soluble TRAIL-R polypeptides, separated by peptide linkers.

Another method for preparing oligomeric TRAIL-R involves use of a leucine zipper. Leucine zipper domains are peptides that promote oligomerization of the proteins in which they are found. Leucine zippers were originally identified in several DNA-binding proteins (Landschulz et al., *Science* 240:1759, 1988), and have since been found in a variety of different proteins. Among the known leucine zippers are naturally occurring peptides and derivatives thereof that dimerize or trimerize. Examples of leucine zipper domains suitable for producing soluble oligomeric proteins are described in PCT application WO 94/10308, the leucine zipper derived from lung surfactant protein D (SPD) described in Hoppe et al. (*FEBS Letters* 344:191, 1994) and U.S. Patent application serial no. 08/446,922, hereby incorporated by reference.

Recombinant fusion proteins comprising a soluble TRAIL-R polypeptide fused to a leucine zipper peptide are expressed in suitable host cells, and the soluble oligomeric TRAIL-R that forms is recovered from the culture supernatant.

Oligomeric TRAIL-R has the property of bivalent, trivalent, etc. binding sites for TRAIL. The above-described fusion proteins comprising Fc moieties (and oligomers formed therefrom) offer the advantage of facile purification by affinity chromatography over Protein A or Protein G columns.

Assays

TRAIL-R proteins (including fragments, variants, oligomers, and other forms of TRAIL-R) may be tested for the ability to bind TRAIL in any suitable assay, such as a conventional binding assay. To illustrate, TRAIL-R may be labeled with a detectable reagent (e.g., a radionuclide, chromophore, enzyme that catalyzes a colorimetric or fluorometric reaction, and the like). The labeled TRAIL-R is contacted with cells expressing TRAIL. The cells then are washed to remove unbound labeled TRAIL-R, and the presence of cell-bound label is determined by a suitable technique, chosen according to the nature of the label.

One example of a binding assay procedure is as follows. A recombinant expression vector containing TRAIL cDNA is constructed, e.g., as described in in PCT application WO 97/01633, hereby incorporated by reference. DNA and amino acid sequence information for human and mouse TRAIL is presented in WO 97/01633. TRAIL comprises an N-terminal cytoplasmic domain, a transmembrane region, and a C-terminal extracellular domain. CV1-EBNA-1 cells in 10 $cm^2$ dishes are transfected with the recombinant expression vector. CV-1/EBNA-1 cells (ATCC CRL 10478) constitutively express EBV nuclear antigen-1 driven from the CMV immediate-early enhancer/promoter. CV1-EBNA-1 was derived from the African Green Monkey kidney cell line CV-1 (ATCC CCL 70), as described by McMahan et al. (*EMBO J.* 10:2821, 1991).

The transfected cells are cultured for 24 hours, and the cells in each dish then are split into a 24-well plate. After culturing an additional 48 hours, the transfected cells (about 4 x $10^4$ cells/well) are washed with BM-NFDM, which is binding medium (RPMI 1640 containing 25 mg/ml bovine serum albumin, 2 mg/ml sodium azide, 20 mM Hepes pH 7.2) to which 50 mg/ml nonfat dry milk has been added. The cells then are incubated for 1 hour at 37°C with various concentrations of a soluble TRAIL-R/Fc fusion protein. Cells then are washed and incubated with a constant saturating

concentration of a $^{125}$I-mouse anti-human IgG in binding medium, with gentle agitation for 1 hour at 37°C. After extensive washing, cells are released *via* trypsinization.

The mouse anti-human IgG employed above is directed against the Fc region of human IgG and can be obtained from Jackson Immunoresearch Laboratories, Inc., West Grove, PA. The antibody is radioiodinated using the standard chloramine-T method. The antibody will bind to the Fc portion of any TRAIL-R/Fc protein that has bound to the cells. In all assays, non-specific binding of $^{125}$I-antibody is assayed in the absence of TRAIL-R/Fc, as well as in the presence of TRAIL-R/Fc and a 200-fold molar excess of unlabeled mouse anti-human IgG antibody.

Cell-bound $^{125}$I-antibody is quantified on a Packard Autogamma counter. Affinity calculations (Scatchard, *Ann. N.Y. Acad. Sci.* 51:660, 1949) are generated on RS/1 (BBN Software, Boston, MA) run on a Microvax computer.

Another type of suitable binding assay is a competitive binding assay. To illustrate, biological activity of a TRAIL-R variant may be determined by assaying for the variant's ability to compete with a native TRAIL-R for binding to TRAIL.

Competitive binding assays can be performed by conventional methodology. Reagents that may be employed in competitive binding assays include radiolabeled TRAIL-R and intact cells expressing TRAIL (endogenous or recombinant) on the cell surface. For example, a radiolabeled soluble TRAIL-R fragment can be used to compete with a soluble TRAIL-R variant for binding to cell surface TRAIL. Instead of intact cells, one could substitute a soluble TRAIL/Fc fusion protein bound to a solid phase through the interaction of Protein A or Protein G (on the solid phase) with the Fc moiety. Chromatography columns that contain Protein A and Protein G include those available from Pharmacia Biotech, Inc., Piscataway, NJ. Another type of competitive binding assay utilizes radiolabeled soluble TRAIL, such as a soluble TRAIL/Fc fusion protein, and intact cells expressing TRAIL-R. Qualitative results can be obtained by competitive autoradiographic plate binding assays, while Scatchard plots (Scatchard, *Ann. N.Y. Acad. Sci.* 51:660, 1949) may be utilized to generate quantitative results.

Another type of assay for biological activity involves testing a TRAIL-R polypeptide for the ability to block TRAIL-mediated apoptosis of target cells, such as the human leukemic T-cell line known as Jurkat cells, for example. TRAIL-mediated apoptosis of the cell line designated Jurkat clone E6-1 (ATCC TIB 152) is demonstrated in assay procedures described in PCT application WO 97/01633, hereby incorporated by reference.

Uses of TRAIL-R

Uses of TRAIL-R include, but are not limited to, the following. Certain of these uses of TRAIL-R flow from its ability to bind TRAIL.

TRAIL-R finds use as a protein purification reagent. TRAIL-R polypeptides may be attached to a solid support material and used to purify TRAIL proteins by affinity chromatography. In particular embodiments, a TRAIL-R polypeptide (in any form described herein that is capable of binding TRAIL) is attached to a solid support by conventional procedures. As one example, chromatography columns containing functional groups that will react with functional groups on amino acid side chains of proteins are available (Pharmacia Biotech, Inc., Piscataway, NJ). In an alternative, a TRAIL-R/Fc protein is attached to Protein A- or Protein G-containing chromatography columns through interaction with the Fc moiety.

TRAIL-R proteins also find use in measuring the biological activity of TRAIL proteins in terms of their binding affinity for TRAIL-R. TRAIL-R proteins thus may be employed by those conducting "quality assurance" studies, e.g., to monitor shelf life and stability of TRAIL protein under different conditions. To illustrate, TRAIL-R may be employed in a binding affinity study to measure the biological activity of a TRAIL protein that has been stored at different temperatures, or produced in different cell types. TRAIL-R also may be used to determine whether biological activity is retained after modification of a TRAIL protein (e.g., chemical modification, truncation, mutation, etc.). The binding affinity of the modified TRAIL protein for TRAIL-R is compared to that of an unmodified TRAIL protein to detect any adverse impact of the modifications on biological activity of TRAIL. The biological activity of a TRAIL protein thus can be ascertained before it is used in a research study, for example.

TRAIL-R also finds use in purifying or identifying cells that express TRAIL on the cell surface. TRAIL-R polypeptides are bound to a solid phase such as a column chromatography matrix or a similar suitable substrate. For example, magnetic microspheres can be coated with TRAIL-R and held in an incubation vessel through a magnetic field. Suspensions of cell mixtures containing TRAIL-expressing cells are contacted with the solid phase having TRAIL-R thereon. Cells expressing TRAIL on the cell surface bind to the fixed TRAIL-R, and unbound cells then are washed away.

Alternatively, TRAIL-R can be conjugated to a detectable moiety, then incubated with cells to be tested for TRAIL expression. After incubation, unbound labeled TRAIL-R is removed and the presence or absence of the detectable moiety on the cells is determined.

In a further alternative, mixtures of cells suspected of containing TRAIL$^+$ cells are incubated with biotinylated TRAIL-R. Incubation periods are typically at least one

hour in duration to ensure sufficient binding. The resulting mixture then is passed through a column packed with avidin-coated beads, whereby the high affinity of biotin for avidin provides binding of the desired cells to the beads. Procedures for using avidin-coated beads are known (see Berenson, et al. *J. Cell. Biochem.*, 10D:239, 1986). Washing to remove unbound material, and the release of the bound cells, are performed using conventional methods.

TRAIL-R polypeptides also find use as carriers for delivering agents attached thereto to cells bearing TRAIL. Cells expressing TRAIL include those identified in Wiley et al. (*Immunity*, 3:673-682, 1995). TRAIL-R proteins thus can be used to deliver diagnostic or therapeutic agents to such cells (or to other cell types found to express TRAIL on the cell surface) in *in vitro* or *in vivo* procedures.

Detectable (diagnostic) and therapeutic agents that may be attached to a TRAIL-R polypeptide include, but are not limited to, toxins, other cytotoxic agents, drugs, radionuclides, chromophores, enzymes that catalyze a colorimetric or fluorometric reaction, and the like, with the particular agent being chosen according to the intended application. Among the toxins are ricin, abrin, diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A, ribosomal inactivating proteins, mycotoxins such as trichothecenes, and derivatives and fragments (e.g., single chains) thereof. Radionuclides suitable for diagnostic use include, but are not limited to, $^{123}I$, $^{131}I$, $^{99m}Tc$, $^{111}In$, and $^{76}Br$. Examples of radionuclides suitable for therapeutic use are $^{131}I$, $^{211}At$, $^{77}Br$, $^{186}Re$, $^{188}Re$, $^{212}Pb$, $^{212}Bi$, $^{109}Pd$, $^{64}Cu$, and $^{67}Cu$.

Such agents may be attached to the TRAIL-R by any suitable conventional procedure. TRAIL-R, being a protein, comprises functional groups on amino acid side chains that can be reacted with functional groups on a desired agent to form covalent bonds, for example. Alternatively, the protein or agent may be derivatized to generate or attach a desired reactive functional group. The derivatization may involve attachment of one of the bifunctional coupling reagents available for attaching various molecules to proteins (Pierce Chemical Company, Rockford, Illinois). A number of techniques for radiolabeling proteins are known. Radionuclide metals may be attached to TRAIL-R by using a suitable bifunctional chelating agent, for example.

Conjugates comprising TRAIL-R and a suitable diagnostic or therapeutic agent (preferably covalently linked) are thus prepared. The conjugates are administered or otherwise employed in an amount appropriate for the particular application.

Another use of the protein of the present invention is as a research tool for studying the biological effects that result from inhibiting TRAIL/TRAIL-R interactions

on different cell types. TRAIL-R polypeptides also may be employed in *in vitro* assays for detecting TRAIL or TRAIL-R or the interactions thereof.

TRAIL-R may be employed in inhibiting a biological activity of TRAIL, in *in vitro* or *in vivo* procedures. A purified TRAIL-R polypeptide may be used to inhibit binding of TRAIL to endogenous cell surface TRAIL-R. Biological effects that result from the binding of TRAIL to endogenous receptors thus are inhibited. Various forms of TRAIL-R may be employed, including, for example, the above-described TRAIL-R fragments, oligomers, derivatives, and variants that are capable of binding TRAIL. In one embodiment, a soluble TRAIL-R is employed to inhibit a biological activity of TRAIL, e.g., to inhibit TRAIL-mediated apoptosis of particular cells.

TRAIL-R may be administered to a mammal to treat a TRAIL-mediated disorder. Such TRAIL-mediated disorders include conditions caused (directly or indirectly) or exacerbated by TRAIL.

TRAIL-R may be useful for treating thrombotic microangiopathies. One such disorder is thrombotic thrombocytopenic purpura (TTP) (Kwaan, H.C., *Semin. Hematol.*, 24:71, 1987; Thompson et al., *Blood*, 80:1890, 1992). Increasing TTP-associated mortality rates have been reported by the U.S. Centers for Disease Control (Torok et al., *Am. J. Hematol.* 50:84, 1995).

Plasma from patients afflicted with TTP (including $HIV^+$ and $HIV^-$ patients) induces apoptosis of human endothelial cells of dermal microvascular origin, but not large vessel origin (Laurence et al., *Blood*, 87:3245, April 15, 1996). Plasma of TTP patients thus is thought to contain one or more factors that directly or indirectly induce apoptosis. As described in PCT application WO 97/01633 (hereby incorporated by reference), TRAIL is present in the serum of TTP patients, and may play a role in inducing apoptosis of microvascular endothelial cells.

Another thrombotic microangiopathy is hemolytic-uremic syndrome (HUS) (Moake, J.L., *Lancet*, 343:393, 1994; Melnyk et al., (*Arch. Intern. Med.*, 155:2077, 1995; Thompson et al., *supra*). One embodiment of the invention is directed to use of TRAIL-R to treat the condition that is often referred to as "adult HUS" (even though it can strike children as well). A disorder known as childhood/diarrhea-associated HUS differs in etiology from adult HUS.

Other conditions characterized by clotting of small blood vessels may be treated using TRAIL-R. Such conditions include but are not limited to the following. Cardiac problems seen in about 5-10% of pediatric AIDS patients are believed to involve clotting of small blood vessels. Breakdown of the microvasculature in the heart has

been reported in multiple sclerosis patients. As a further example, treatment of systemic lupus erythematosus (SLE) is contemplated.

In one embodiment, a patient's blood or plasma is contacted with TRAIL-R *ex vivo*. The TRAIL-R may be bound to a suitable chromatography matrix by conventional procedures. The patient's blood or plasma flows through a chromatography column containing TRAIL-R bound to the matrix, before being returned to the patient. The immobilized receptor binds TRAIL, thus removing TRAIL protein from the patient's blood.

Alternatively, TRAIL-R may be administered *in vivo* to a patient afflicted with a thrombotic microangiopathy. In one embodiment, a soluble form of TRAIL-R is administered to the patient.

The present invention thus provides a method for treating a thrombotic microangiopathy, involving use of an effective amount of TRAIL-R. A TRAIL-R polypeptide may be employed in *in vivo* or *ex vivo* procedures, to inhibit TRAIL-mediated damage to (e.g., apoptosis of) microvascular endothelial cells.

TRAIL-R may be employed in conjunction with other agents useful in treating a particular disorder. In an *in vitro* study reported by Laurence et al. (*Blood* 87:3245, 1996), some reduction of TTP plasma-mediated apoptosis of microvascular endothelial cells was achieved by using an anti-Fas blocking antibody, aurintricarboxylic acid, or normal plasma depleted of cryoprecipitate.

Thus, a patient may be treated with an agent that inhibits Fas-ligand-mediated apoptosis of endothelial cells, in combination with an agent that inhibits TRAIL-mediated apoptosis of endothelial cells. In one embodiment, TRAIL-R and an anti-FAS blocking antibody are both administered to a patient afflicted with a disorder characterized by thrombotic microangiopathy, such as TTP or HUS. Examples of blocking monoclonal antibodies directed against Fas antigen (CD95) are described in PCT application publication number WO 95/10540, hereby incorporated by reference.

TRAIL-R of the present invention may be used in developing treatments for any disorder mediated (directly or indirectly) by defective, or insufficient amounts of, TRAIL-R. TRAIL-R polypeptides may be administered to a mammal afflicted with such a disorder.

Compositions comprising an effective amount of a TRAIL-R polypeptide of the present invention, in combination with other components such as a physiologically acceptable diluent, carrier, or excipient, are provided herein. TRAIL-R can be formulated according to known methods used to prepare pharmaceutically useful compositions. TRAIL-R can be combined in admixture, either as the sole active

material or with other known active materials suitable for a given indication, with pharmaceutically acceptable diluents (e.g., saline, Tris-HCl, acetate, and phosphate buffered solutions), preservatives (e.g., thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable formulations for pharmaceutical compositions include those described in *Remington's Pharmaceutical Sciences*, 16th ed. 1980, Mack Publishing Company, Easton, PA.

In addition, such compositions can contain TRAIL-R complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of TRAIL-R, and are thus chosen according to the intended application. TRAIL-R expressed on the surface of a cell may find use, as well.

Compositions of the present invention may contain a TRAIL-R polypeptide in any form described herein, such as native proteins, variants, derivatives, oligomers, and biologically active fragments. In particular embodiments, the composition comprises a soluble TRAIL-R polypeptide or an oligomer comprising soluble TRAIL-R polypeptides.

TRAIL-R can be administered in any suitable manner, e.g., topically, parenterally, or by inhalation. The term "parenteral" includes injection, e.g., by subcutaneous, intravenous, or intramuscular routes, also including localized administration, e.g., at a site of disease or injury. Sustained release from implants is also contemplated. One skilled in the pertinent art will recognize that suitable dosages will vary, depending upon such factors as the nature of the disorder to be treated, the patient's body weight, age, and general condition, and the route of administration. Preliminary doses can be determined according to animal tests, and the scaling of dosages for human administration are performed according to art-accepted practices.

Antibodies

Antibodies that are immunoreactive with TRAIL-R polypeptides are provided herein. Such antibodies specifically bind TRAIL-R, in that the antibodies bind to TRAIL-R *via* the antigen-binding sites of the antibody (as opposed to non-specific binding).

The TRAIL-R protein prepared as described in example 1 may be employed as an immunogen in producing antibodies immunoreactive therewith. Alternatively,

another form of TRAIL-R, such as a fragment or fusion protein, is employed as the immunogen.

Polyclonal and monoclonal antibodies may be prepared by conventional techniques. See, for example, *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Kennet et al. (eds.), Plenum Press, New York (1980); and *Antibodies: A Laboratory Manual*, Harlow and Land (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1988). Production of monoclonal antibodies directed against TRAIL-R is further illustrated in example 4.

Antigen-binding fragments of such antibodies, which may be produced by conventional techniques, are also encompassed by the present invention. Examples of such fragments include, but are not limited to, Fab and $F(ab')_2$ fragments. Antibody fragments and derivatives produced by genetic engineering techniques are also provided.

The monoclonal antibodies of the present invention include chimeric antibodies, e.g., humanized versions of murine monoclonal antibodies. Such humanized antibodies may be prepared by known techniques, and offer the advantage of reduced immunogenicity when the antibodies are administered to humans. In one embodiment, a humanized monoclonal antibody comprises the variable region of a murine antibody (or just the antigen binding site thereof) and a constant region derived from a human antibody. Alternatively, a humanized antibody fragment may comprise the antigen binding site of a murine monoclonal antibody and a variable region fragment (lacking the antigen-binding site) derived from a human antibody. Procedures for the production of chimeric and further engineered monoclonal antibodies include those described in Riechmann et al. (*Nature 332*:323, 1988), Liu et al. (*PNAS 84*:3439, 1987), Larrick et al. (*Bio/Technology* 7:934, 1989), and Winter and Harris (*TIPS 14*:139, May, 1993).

Among the uses of the antibodies is use in assays to detect the presence of TRAIL-R polypeptides, either *in vitro* or *in vivo*. The antibodies also may be employed in purifying TRAIL-R proteins by immunoaffinity chromatography.

Those antibodies that additionally can block binding of TRAIL-R to TRAIL may be used to inhibit a biological activity that results from such binding. Such blocking antibodies may be identified using any suitable assay procedure, such as by testing antibodies for the ability to inhibit binding of TRAIL to cells expressing TRAIL-R. Examples of such cells are the Jurkat cells and PS1 cells described in example 2 below. Alternatively, blocking antibodies may be identified in assays for the ability to inhibit a biological effect that results from binding of TRAIL to target cells. Antibodies

may be assayed for the ability to inhibit TRAIL-mediated lysis of Jurkat cells, for example.

Such an antibody may be employed in an *in vitro* procedure, or administered *in vivo* to inhibit a TRAIL-R-mediated biological activity. Disorders caused or exacerbated (directly or indirectly) by the interaction of TRAIL with cell surface TRAIL receptor thus may be treated. A therapeutic method involves *in vivo* administration of a blocking antibody to a mammal in an amount effective in inhibiting a TRAIL-mediated biological activity. Disorders caused or exacerbated by TRAIL, directly or indirectly, are thus treated. Monoclonal antibodies are generally preferred for use in such therapeutic methods. In one embodiment, an antigen-binding antibody fragment is employed.

A blocking antibody directed against TRAIL-R may be substituted for TRAIL-R in the above-described method of treating thrombotic microangiopathy, e.g., in treating TTP or HUS. The antibody is administered *in vivo*, to inhibit TRAIL-mediated damage to (e.g., apoptosis of) microvascular endothelial cells.

Compositions comprising an antibody that is directed against TRAIL-R, and a physiologically acceptable diluent, excipient, or carrier, are provided herein. Suitable components of such compositions are as described above for compositions containing TRAIL-R proteins.

Also provided herein are conjugates comprising a detectable (e.g., diagnostic) or therapeutic agent, attached to an antibody directed against TRAIL-R. Examples of such agents are presented above. The conjugates find use in *in vitro* or *in vivo* procedures.

Nucleic Acids and Uses Thereof

The present invention provides TRAIL-R nucleic acids. Such nucleic acids include, but are not limited to, DNA encoding the peptides described in example 2. Such DNAs can be identified from knowledge of the genetic code. One embodiment of the invention is directed to fragments of TRAIL-R nucleotide sequences comprising at least about 17 contiguous nucleotides of a TRAIL-R DNA sequence. Nucleic acids provided herein include DNA and RNA complements of said fragments, along with both single-stranded and double-stranded forms of the TRAIL-R DNA.

Among the uses of TRAIL-R nucleic acid fragments is use as probes or primers. Using knowledge of the genetic code in combination with the amino acid sequences set forth in example 2, sets of degenerate oligonucleotides can be prepared.

Such oligonucleotides find use as primers, e.g., in polymerase chain reactions (PCR), whereby TRAIL-R DNA fragments are isolated and amplified.

The following examples are provided to further illustrate particular embodiments of the invention, and are not to be construed as limiting the scope of the present invention.

## EXAMPLE 1: Purification of TRAIL-R Protein

A human TRAIL receptor (TRAIL-R) protein was prepared by the following procedure. Trail-R was isolated from the cell membranes of Jurkat cells, a human acute T leukemia cell line. Jurkat cells were chosen because a specific band can be affinity precipitated from surface-biotinylated Jurkat cells, using Flag®-TRAIL covalently coupled to affi-gel beads (Biorad Laboratories, Richmond, CA). The precipitated band has a molecular weight of about 52 kD. A minor specific band of about 42 kD also was present, possibly accounting for a proteolytic breakdown product or a less glycosylated form of TRAIL-R.

Approximately 50 billion Jurkat cells were harvested by centrifugation (80 ml of cell pellet), washed once with PBS, then shock frozen on liquid nitrogen. Plasma membranes were isolated according to method number three described in Maeda et al., *Biochim. et Biophys. Acta*, 731:115, 1983; hereby incorporated by reference) with five modifications:

1. The following protease inhibitors were included in all solutions at the indicated concentrations: Aprotinin, 150 nM; EDTA, 5 mM; Leupeptin, 1 μM; pA-PMSF, 20 μM; Pefabloc, 500 μM; Pepstatin A, 1 μM; PMSF, 500 μM.
2. Dithiothreitol was not used.
3. DNAase was not used in the homogenization solution.
4. 1.25 ml of homogenization buffer was used per ml of cell pellet.
5. The homogenization was accomplished by five passages through a ground glass dounce homogenizer.

After isolation of the cell membranes, proteins were solubilized by resuspending the isolated membranes in 50 ml PBS containing 1% octylglucoside and all of the above mentioned protease inhibitors at the above indicated concentrations. The resulting solution was then repeatedly vortexed during a thirty-minute incubation at 4°C. The solution was then centrifuged at 20,000 rpm in an SW28 rotor in an LE-80

Beckman ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) at 4°C for 30 minutes to obtain the supernatant (the membrane extract).

Chromatography

The first step of purification of TRAIL-R out of the membrane extract prepared above was affinity chromatography. The membrane extract was first applied to an anti-Flag® M2 affi-gel column (10 mg of monoclonal antibody M2 coupled to 2 ml of Affi-gel beads) to adsorb any nonspecifically binding material. The flow-through was then applied to a Flag®-TRAIL affi-gel column (10 mg of recombinant protein coupled to 1 ml of affi-gel beads).

The Affi-gel support is an N-hydroxysuccinimide ester of a derivatized, crosslinked agarose gel bead (available from Biorad Laboratories, Richmond, CA). As discussed above, the Flag® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, provides an epitope reversibly bound by specific monoclonal antibodies, enabling rapid assay and facile purification of expressed recombinant protein. M2 is a monoclonal antibody that binds Flag®. Monoclonal antibodies that bind the Flag® peptide, as well as other reagents for preparing and using Flag® fusion proteins, are available from Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Connecticut. Preparation of Flag®-TRAIL fusion proteins (comprising Flag® fused to a soluble TRAIL polypeptide) is further described in PCT application WO 97/01633, hereby incorporated by reference.

The column was washed with 25 ml of each of the following buffers, in the order indicated:

1. PBS containing 1% octylglucoside
2. PBS
3. PBS containing an additional 200 mM NaCl
4. PBS

The bound material was eluted with 50 mM Na Citrate (pH 3) in 1 ml fractions and immediately neutralized with 300 μl of 1 M Tris-HCl (pH 8.5) per fraction. The TRAIL-binding activity of each fraction was determined by a TRAIL-R-specific ELISA as described below. Fractions with high TRAIL-binding activity were pooled, brought to 0.1 % Trifluoroacetic acid (TFA), and subsequently chromatographed on a capillary reversed-phase HPLC column [500 μm internal diameter X 25 cm fused silicone capillary column packed with 5 μm Vydac $C_4$ material (Vydac, Hesperia, CA)] using a linear gradient (2% per minute) from 0% to 100% acetonitrile in water containing 0.1%

TFA. Fractions containing high TRAIL-binding activity are then determined as above, pooled, and, if desired, lyophylized.

TRAIL-R-specific ELISA:

Serial dilutions of TRAIL-R-containing samples (in 50 mM $NaHCO_3$, brought to pH 9 with NaOH) were coated onto Linbro/Titertek 96 well flat bottom E.I.A. microtitration plates (ICN Biomedicals Inc., Aurora, OH) at 100 µl/well. After incubation at 4°C for 16 hours, the wells were washed six times with 200 µl PBS containing 0.05% Tween-20 (PBS-Tween). The wells were then incubated with Flag®-TRAIL at 1 µg/ml in PBS-Tween with 5% fetal calf serum (FCS) for 90 minutes (100 µl per well), followed by washing as above. Next, each well was incubated with the anti-Flag® monoclonal antibody M2 at 1 µg/ml in PBS-Tween containing 5% FCS for 90 minutes (100 µl per well), followed by washing as above. Subsequently, wells were incubated with a polyclonal goat anti-mIgG1-specific horseradish peroxidase-conjugated antibody (a 1:5000 dilution of the commercial stock in PBS-Tween containing 5% FCS) for 90 minutes (100 µl per well). The HRP-conjugated antibody was obtained from Southern Biotechnology Associates, Inc., Birmingham, Alabama. Wells then were washed six times, as above.

For development of the ELISA, a substrate mix [100 µl per well of a 1:1 premix of the TMB Peroxidase Substrate and Peroxidase Solution B (Kirkegaard Perry Laboratories, Gaithersburg, Maryland)] was added to the wells. After sufficient color reaction, the enzymatic reaction was terminated by addition of 2 N $H_2SO_4$ (50 µl per well). Color intensity (indicating TRAIL-binding activity) was determined by measuring extinction at 450 nm on a V Max plate reader (Molecular Devices, Sunnyvale, CA).

## EXAMPLE 2: Amino Acid Sequence

(a) TRAIL-R purified from Jurkat cells

TRAIL-R protein isolated from Jurkat cells was digested with trypsin, using conventional procedures. Amino acid sequence analysis was conducted on one of the peptide fragments produced by the tryptic digest. The fragment was found to contain the sequence:

VPANEGD

(b) TRAIL-R purified from PS-1 cells

TRAIL-R protein was also isolated from PS-1 cells. PS-1 is a human B cell line that spontaneously arose after lethal irradiation of human peripheral blood lymphocytes (PBLs). The TRAIL-R protein was digested with trypsin, using conventional procedures. Amino acid sequence analysis was conducted on peptide fragments that resulted from the tryptic digest. One of the fragments was found to contain the sequence:

VPANEGD

Two other fragments were found to contain the following amino acid sequences, respectively:

VCEC
SGEVELSSV

## EXAMPLE 3: DNA and Amino Acid Sequences

The amino acid sequence of additional tryptic digest peptide fragments of TRAIL-R was determined. Degenerate oligonucleotides, based upon the amino acid sequence of two of the peptides, were prepared. A TRAIL-R DNA fragment was isolated and amplified by polymerase chain reaction (PCR), using the degenerate oligonucleotides as 5' and 3' primers. The PCR was conducted according to conventional procedures, using cDNA derived from the PS-1 cell line described in example 2 as the template. The nucleotide sequence of the isolated TRAIL-R DNA fragment, and the amino acid sequence encoded thereby, are presented in Figure 1.

The amino acid sequence in Figure 1 bears significant homology to the so-called death domains found in certain other receptors. The cytoplasmic region of Fas and TNF receptor type I each contain a death domain, which is associated with transduction of an apoptotic signal (Tartaglia et al. *Cell* 74:845, 1993; Itoh and Nagata, *J. Biol. Chem.* 268:10932, 1993). Thus, the sequence presented in Figure 1 is believed to be found within the cytoplasmic domain of TRAIL-R.

## EXAMPLE 4: Monoclonal Antibodies That Bind TRAIL-R

This example illustrates a method for preparing monoclonal antibodies that bind TRAIL-R. Suitable immunogens that may be employed in generating such antibodies include, but are not limited to, purified TRAIL-R protein or an immunogenic fragment

thereof such as the extracellular domain, or fusion proteins containing TRAIL-R (e.g., a soluble TRAIL-R/Fc fusion protein).

Purified TRAIL-R can be used to generate monoclonal antibodies immunoreactive therewith, using conventional techniques such as those described in U.S. Patent 4,411,993. Briefly, mice are immunized with TRAIL-R immunogen emulsified in complete Freund's adjuvant, and injected in amounts ranging from 10-100 μg subcutaneously or intraperitoneally. Ten to twelve days later, the immunized animals are boosted with additional TRAIL-R emulsified in incomplete Freund's adjuvant. Mice are periodically boosted thereafter on a weekly to bi-weekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision to test for TRAIL-R antibodies by dot blot assay, ELISA (Enzyme-Linked Immunosorbent Assay) or inhibition of TRAIL binding.

Following detection of an appropriate antibody titer, positive animals are provided one last intravenous injection of TRAIL-R in saline. Three to four days later, the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line, e.g., NS1 or preferably P3x63Ag8.653 (ATCC CRL 1580). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells are screened by ELISA for reactivity against purified TRAIL-R by adaptations of the techniques disclosed in Engvall et al., *Immunochem.* 8:871, 1971 and in U.S. Patent 4,703,004. A preferred screening technique is the antibody capture technique described in Beckmann et al., (*J. Immunol.* 144:4212, 1990) Positive hybridoma cells can be injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-TRAIL-R monoclonal antibodies. Alternatively, hybridoma cells can be grown *in vitro* in flasks or roller bottles by various techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to Protein A or Protein G can also be used, as can affinity chromatography based upon binding to TRAIL-R.

What is claimed is:

1. A purified TRAIL receptor (TRAIL-R) polypeptide that is capable of binding TRAIL, wherein the TRAIL-R is characterized as comprising the amino acid sequence VPANEGD.

2. A TRAIL-R polypeptide of claim 1, wherein said polypeptide is further characterized by a molecular weight of about 50 to 55 kilodaltons.

3. A purified TRAIL-R polypeptide that is capable of binding TRAIL, wherein the TRAIL-R is characterized as comprising at least one of the amino acid sequences selected from the group consisting of VPANEGD, VCEC and SGEVELSSV.

4. A TRAIL-R polypeptide of claim 3, wherein said polypeptide is further characterized by a molecular weight of about 50 to 55 kilodaltons.

5. A TRAIL-R polypeptide of claim 3, wherein said polypeptide is further characterized by comprising the amino acid sequence ETLRQCFDDFADLVPFDS WEPLMRKLGLMDNEIKVAKAEAAGHRDTLXTML.

6. A TRAIL-R polypeptide of claim 5, wherein said polypeptide is further characterized by a molecular weight of about 50 to 55 kilodaltons

7. A purified TRAIL-R polypeptide, wherein said polypeptide is a fragment of the TRAIL-R polypeptide of claim 1.

8. A purified TRAIL-R polypeptide, wherein said polypeptide is a fragment of the TRAIL-R polypeptide of claim 3.

9. A purified TRAIL-R polypeptide, wherein said polypeptide is a fragment of the TRAIL-R polypeptide of claim 5.

10. A TRAIL-R polypeptide of claim 7, wherein said fragment is a soluble TRAIL-R polypeptide.

11. A TRAIL-R polypeptide of claim 8, wherein said fragment is a soluble TRAIL-R polypeptide.

12. A TRAIL-R polypeptide of claim 9, wherein said fragment is a soluble TRAIL-R polypeptide.

13. An oligomer comprising from two to four TRAIL-R polypeptides of claim 1, 3, 7, 8, 10, or 11.

14. An oligomer of claim 13, wherein said oligomer is a dimer comprising two soluble TRAIL-R/Fc fusion proteins.

15. A composition comprising a TRAIL-R polypeptide of claim 1, 3, 7, 8, 10, or 11, and a physiologically acceptable diluent, excipient, or carrier.

16. A composition comprising an oligomer of claim 13 and a physiologically acceptable diluent, excipient, or carrier.

17. An isolated TRAIL-R DNA, wherein said DNA comprises the nucleotide sequence presented in Figure 1.

18. An antibody that is directed against a TRAIL-R polypeptide of claim 1, 3, or 5.

19. An antibody of claim 18, wherein the antibody is a monoclonal antibody.

# FIGURE 1

```
    CTGAGACTCTGAGACAGTGCTTCGATGACTTTGCAGACTTGGTGCCCTTTGACTCCTGGG
  1 ---------+---------+---------+---------+---------+---------+ 60
    GACTCTGAGACTCTGTCACGAAGCTACTGAAACGTCTGAACCACGGGAAACTGAGGACCC

     E  T  L  R  Q  C  F  D  D  F  A  D  L  V  P  F  D  S  W  E -

    AGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGG
 61 ---------+---------+---------+---------+---------+---------+ 120
    TCGGCGAGTACTCCTTCAACCCGGAGTACCTGTTACTCTATTTCCACCGATTTCGACTCC

     P  L  M  R  K  L  G  L  M  D  N  E  I  K  V  A  K  A  E  A -

    CAGCGGGCCACAGGGACACCTTGTNCACNATGCTGAT
121 ---------+---------+---------+------- 157
    GTCGCCCGGTGTCCCTGTGGAACANGTGNTACGACTA

     A  G  H  R  D  T  L  X  T  M  L    -
```

# EXHIBIT F

PATENT APPLICATION SERIAL NO. 08/799861

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
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PTO-1556
(5/87)

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | | |
|---|---|---|
| In the Application of: | Charles Rauch and Henning Walczak | Docket No. 2625 |
| Serial No.: | -- to be assigned -- | |
| Filing Date: | February 13, 1997 | |
| For: | RECEPTOR THAT BINDS TRAIL | |

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Specification, claims and abstract (22 pages)

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February 13, 1997

BOX PATENT APPLICATION
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Sir:

Re: Patent Application of Charles Rauch and Henning Walczak

Title: RECEPTOR THAT BINDS TRAIL

Attorney Docket No.: 2625



We transmit herewith for filing the above-identified patent application comprising the following documents

( X ) Certificate of Mailing by Express Mail pursuant to 37 C.F.R. §1.10. A filing date for the application in accordance with 37 C.F.R. §1.10 is requested.

( X ) Patent Specification and Claims, including Abstract ( 22 pages)

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## ABSTRACT OF THE DISCLOSURE

The invention is directed to a protein designated TRAIL receptor, which binds the protein known as TNF-Related Apoptosis-Inducing Ligand (TRAIL). The TRAIL receptor finds use in purifying TRAIL or inhibiting activities thereof. Antibodies that are immunoreactive with TRAIL-R are also provided.

A/N Ltr

08/799861

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Immunex Corporation 2625

TITLE

# RECEPTOR THAT BINDS TRAIL

## BACKGROUND OF THE INVENTION

A protein known as TNF-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family of ligands (Wiley et al., *Immunity*, 3:673-682, 1995). TRAIL has demonstrated the ability to induce apoptosis of certain transformed cells, including a number of different types of cancer cells as well as virally infected cells (PCT application WO 97/01633 and Wiley et al., *supra*).

Identification of receptor protein(s) that bind TRAIL would prove useful in further study of the biological activities of TRAIL. However, prior to the present invention, no receptor for TRAIL had been reported.

## SUMMARY OF THE INVENTION

The present invention is directed to a novel protein designated TRAIL receptor (TRAIL-R). TRAIL-R binds to a protein known as TNF-related apoptosis-inducing ligand (TRAIL). Antibodies that are immunoreactive with TRAIL-R are also provided.

## DETAILED DESCRIPTION OF THE INVENTION

A novel protein designated TRAIL receptor (TRAIL-R) is provided herein. TRAIL-R binds to the cytokine designated TNF-related apoptosis-inducing ligand (TRAIL). Certain uses of TRAIL-R flow from this ability to bind TRAIL, as discussed further below. TRAIL-R finds use in inhibiting biological activities of TRAIL, or in purifying TRAIL by affinity chromatography, for example.

TRAIL-R protein or immunogenic fragments thereof may be employed as immunogens to generate antibodies that are immunoreactive therewith. In one embodiment of the invention, the antibodies are monoclonal antibodies.

A human TRAIL-R protein was purified as described in example 1. In example 2, amino acid sequence information derived from fragments of TRAIL-R is presented. One embodiment of the invention is directed to a purified human TRAIL-R protein that is capable of binding TRAIL, wherein the TRAIL-R is characterized as comprising the amino acid sequences VPANEGD, VCEC and SGEVELSSV. Also provided are

TRAIL-R fragments comprising only one or two of the characterizing amino acid sequences.

In one embodiment, the protein is further characterized by a molecular weight within the range of about 50 to 55 kilodaltons, which is the molecular weight determined for a preparation of native, full length, human TRAIL-R. Molecular weight can be determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The skilled artisan will recognize that the molecular weight of particular preparations of TRAIL-R protein may differ, according to such factors as the degree of glycosylation. The glycosylation pattern of a particular preparation of TRAIL-R may vary according to the type of cells in which the protein is expressed, for example. Further, a given preparation may include multiple differentially glycosylated species of the protein. TRAIL-R polypeptides with or without associated native-pattern glycosylation are provided herein.

As used herein, the term "TRAIL-R" refers to a genus of polypeptides that are substantially homologous to the human TRAIL-R protein isolated in example 1. The present invention encompasses TRAIL-R in various forms, either naturally occurring or produced through known techniques. Such forms of TRAIL-R include, but are not limited to, fragments, derivatives, and oligomers of TRAIL-R, as well as fusion proteins containing TRAIL-R or fragments thereof.

TRAIL-R may be modified to create derivatives thereof by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of TRAIL-R may be prepared by linking the chemical moieties to functional groups on TRAIL-R amino acid side chains or at the N-terminus or C-terminus of a TRAIL-R polypeptide. Conjugates comprising diagnostic (detectable) or therapeutic agents attached to TRAIL-R are contemplated herein, as discussed in more detail below.

Other derivatives of TRAIL-R within the scope of this invention include covalent or aggregative conjugates of TRAIL-R polypeptides with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. Examples of fusion proteins are discussed below in connection with TRAIL-R oligomers. Further, TRAIL-R-containing fusion proteins can comprise peptides added to facilitate purification and identification of TRAIL-R. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., *Bio/Technology* 6:1204, 1988. One such peptide is the Flag® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, which is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid

assay and facile purification of expressed recombinant protein. A murine hybridoma designated 4E11 produces a monoclonal antibody that binds the Flag® peptide in the presence of certain divalent metal cations, as described in U.S. Patent 5,011,912, hereby incorporated by reference. The 4E11 hybridoma cell line has been deposited with the American Type Culture Collection under accession no. HB 9259. Monoclonal antibodies that bind the Flag® peptide are available from Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Connecticut.

One embodiment of the invention is directed to TRAIL-R fragments that retain the ability to bind TRAIL. Such fragments may be prepared by any of a number of conventional techniques. Desired peptide fragments may be chemically synthesized. An alternative involves generating TRAIL-R fragments by enzymatic digestion, e.g., by treating the protein with an enzyme known to cleave proteins at sites defined by particular amino acid residues.

Both cell membrane-bound and soluble (secreted) forms of TRAIL-R are provided herein. Soluble TRAIL-R may be identified (and distinguished from non-soluble membrane-bound counterparts) by separating intact cells expressing a TRAIL-R polypeptide from the culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired protein. The presence of TRAIL-R in the medium indicates that the protein was secreted from the cells and thus is a soluble form of the desired protein. Soluble forms of receptor proteins typically lack the transmembrane region that would cause retention of the protein on the cell surface.

Soluble forms of TRAIL-R possess certain advantages over the membrane-bound form of the protein. Purification of the protein from recombinant host cells is facilitated, since the soluble proteins are secreted from the cells. Further, soluble proteins are generally more suitable for certain applications, e.g., for intravenous administration.

Naturally occurring variants of the TRAIL-R protein isolated in example 1 are provided herein. Such variants include, for example, proteins that result from alternate mRNA splicing events or from proteolytic cleavage of the TRAIL-R protein. Alternate splicing of mRNA may, for example, yield a truncated but biologically active TRAIL-R protein, such as a naturally occurring soluble form of the protein. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the TRAIL-R protein (generally from 1-5 terminal amino acids). TRAIL-R proteins in which differences in amino acid sequence are attributable

to genetic polymorphism (allelic variation among individuals producing the protein) are also contemplated herein.

Isolation of a human TRAIL-R protein is described in example 1. TRAIL-R proteins derived from other mammalian species are contemplated, as well.

Example 1 presents one method for purifying a TRAIL-R protein. Jurkat cells are disrupted, and the subsequent purification process includes affinity chromatography (employing a chromatography matrix containing TRAIL), and reversed phase HPLC.

TRAIL-R polypeptides of the present invention may be purified by any suitable alternative procedure, using known protein purification techniques. In one alternative procedure, the chromatography matrix instead comprises an antibody that binds TRAIL-R. Other cell types expressing TRAIL-R (e.g., the PS-1 cells described in example 2) can be substituted for the Jurkat cells. The cells can be disrupted by any of the numerous known techniques, including freeze-thaw cycling, sonication, mechanical disruption, or by use of cell lysing agents.

The desired degree of purity depends on the intended use of the protein. A relatively high degree of purity is desired when the protein is to be administered *in vivo*, for example. Advantageously, TRAIL-R polypeptides are purified such that no protein bands corresponding to other (non-TRAIL-R) proteins are detectable upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It will be recognized by one skilled in the pertinent field that multiple bands corresponding to TRAIL-R protein may be visualized by SDS-PAGE, due to differential glycosylation, differential post-translational processing, and the like. TRAIL-R most preferably is purified to substantial homogeneity, as indicated by a single protein band upon analysis by SDS-PAGE. The protein band may be visualized by silver staining, Coomassie blue staining, or (if the protein is radiolabeled) by autoradiography.

<u>Oligomeric Forms of TRAIL-R</u>

Encompassed by the present invention are oligomers that contain TRAIL-R polypeptides. TRAIL-R oligomers may be in the form of covalently-linked or non-covalently-linked dimers, trimers, or higher oligomers.

One embodiment of the invention is directed to oligomers comprising multiple TRAIL-R polypeptides joined *via* covalent or non-covalent interactions between peptide moieties fused to the TRAIL-R polypeptides. Such peptides may be peptide linkers (spacers), or peptides that have the property of promoting oligomerization. Leucine zippers and certain polypeptides derived from antibodies are among the peptides that

can promote oligomerization of TRAIL-R polypeptides attached thereto, as described in more detail below.

In particular embodiments, the oligomers comprise from two to four TRAIL-R polypeptides. The TRAIL-R moieties of the oligomer may be soluble polypeptides, as described above.

As one alternative, a TRAIL-R oligomer is prepared using polypeptides derived from immunoglobulins. Preparation of fusion proteins comprising certain heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi et al. (*PNAS USA* 88:10535, 1991); Byrn et al. (*Nature* 344:677, 1990); and Hollenbaugh and Aruffo ("Construction of Immunoglobulin Fusion Proteins", in *Current Protocols in Immunology*, Suppl. 4, pages 10.19.1 - 10.19.11, 1992).

One embodiment of the present invention is directed to a TRAIL-R dimer comprising two fusion proteins created by fusing TRAIL-R to the Fc region of an antibody. A gene fusion encoding the TRAIL-R/Fc fusion protein is inserted into an appropriate expression vector. TRAIL-R/Fc fusion proteins are expressed in host cells transformed with the recombinant expression vector, and allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between the Fc moieties to yield divalent TRAIL-R.

Provided herein are fusion proteins comprising a TRAIL-R polypeptide fused to an Fc polypeptide derived from an antibody. DNA encoding such fusion proteins, as well as dimers containing two fusion proteins joined *via* disulfide bonds between the Fc moieties thereof, are also provided. The term "Fc polypeptide" as used herein includes native and mutein forms of polypeptides derived from the Fc region of an antibody. Truncated forms of such polypeptides containing the hinge region that promotes dimerization are also included. One suitable Fc polypeptide, described in PCT application WO 93/10151 (hereby incorporated by reference), is a single chain polypeptide extending from the N-terminal hinge region to the native C-terminus of the Fc region of a human IgG1 antibody. Another useful Fc polypeptide is the Fc mutein described in U.S. Patent 5,457,035 and in Baum et al., (*EMBO J.* 13:3992-4001, 1994). The amino acid sequence of this mutein is identical to that of the native Fc sequence presented in WO 93/10151, except that amino acid 19 has been changed from Leu to Ala, amino acid 20 has been changed from Leu to Glu, and amino acid 22 has been changed from Gly to Ala. The mutein exhibits reduced affinity for Fc receptors.

In other embodiments, TRAIL-R may be substituted for the variable portion of an antibody heavy or light chain. If fusion proteins are made with both heavy and light

chains of an antibody, it is possible to form a TRAIL-R oligomer with as many as four TRAIL-R extracellular regions.

Alternatively, the oligomer is a fusion protein comprising multiple TRAIL-R polypeptides, with or without peptide linkers (spacer peptides). Among the suitable peptide linkers are those described in U.S. Patents 4,751,180 and 4,935,233, which are hereby incorporated by reference. A DNA sequence encoding a desired peptide linker may be inserted between, and in the same reading frame as, the DNA sequences encoding TRAIL-R, using any suitable conventional technique. For example, a chemically synthesized oligonucleotide encoding the linker may be ligated between sequences encoding TRAIL-R. In particular embodiments, a fusion protein comprises from two to four soluble TRAIL-R polypeptides, separated by peptide linkers.

Another method for preparing oligomeric TRAIL-R involves use of a leucine zipper. Leucine zipper domains are peptides that promote oligomerization of the proteins in which they are found. Leucine zippers were originally identified in several DNA-binding proteins (Landschulz et al., *Science* 240:1759, 1988), and have since been found in a variety of different proteins. Among the known leucine zippers are naturally occurring peptides and derivatives thereof that dimerize or trimerize. Examples of leucine zipper domains suitable for producing soluble oligomeric proteins are described in PCT application WO 94/10308, the leucine zipper derived from lung surfactant protein D (SPD) described in Hoppe et al. (*FEBS Letters* 344:191, 1994) and U.S. Patent application serial no. 08/446,922, hereby incorporated by reference. Recombinant fusion proteins comprising a soluble TRAIL-R polypeptide fused to a leucine zipper peptide are expressed in suitable host cells, and the soluble oligomeric TRAIL-R that forms is recovered from the culture supernatant.

Oligomeric TRAIL-R has the property of bivalent, trivalent, etc. binding sites for TRAIL. The above-described fusion proteins comprising Fc moieties (and oligomers formed therefrom) offer the advantage of facile purification by affinity chromatography over Protein A or Protein G columns.

<u>Assays</u>

TRAIL-R proteins (including fragments, variants, oligomers, and other forms of TRAIL-R) may be tested for the ability to bind TRAIL in any suitable assay, such as a conventional binding assay. To illustrate, TRAIL-R may be labeled with a detectable reagent (e.g., a radionuclide, chromophore, enzyme that catalyzes a colorimetric or fluorometric reaction, and the like). The labeled TRAIL-R is contacted with cells expressing TRAIL. The cells then are washed to remove unbound labeled TRAIL-R,

and the presence of cell-bound label is determined by a suitable technique, chosen according to the nature of the label.

One example of a binding assay procedure is as follows. A recombinant expression vector containing TRAIL cDNA is constructed, e.g., as described in in PCT application WO 97/01633, hereby incorporated by reference. DNA and amino acid sequence information for human and mouse TRAIL is presented in WO 97/01633. TRAIL comprises an N-terminal cytoplasmic domain, a transmembrane region, and a C-terminal extracellular domain. CV1-EBNA-1 cells in 10 $cm^2$ dishes are transfected with the recombinant expression vector. CV-1/EBNA-1 cells (ATCC CRL 10478) constitutively express EBV nuclear antigen-1 driven from the CMV immediate-early enhancer/promoter. CV1-EBNA-1 was derived from the African Green Monkey kidney cell line CV-1 (ATCC CCL 70), as described by McMahan et al. (*EMBO J.* 10:2821, 1991).

The transfected cells are cultured for 24 hours, and the cells in each dish then are split into a 24-well plate. After culturing an additional 48 hours, the transfected cells (about 4 x $10^4$ cells/well) are washed with BM-NFDM, which is binding medium (RPMI 1640 containing 25 mg/ml bovine serum albumin, 2 mg/ml sodium azide, 20 mM Hepes pH 7.2) to which 50 mg/ml nonfat dry milk has been added. The cells then are incubated for 1 hour at 37°C with various concentrations of a soluble TRAIL-R/Fc fusion protein. Cells then are washed and incubated with a constant saturating concentration of a $^{125}$I-mouse anti-human IgG in binding medium, with gentle agitation for 1 hour at 37°C. After extensive washing, cells are released *via* trypsinization.

The mouse anti-human IgG employed above is directed against the Fc region of human IgG and can be obtained from Jackson Immunoresearch Laboratories, Inc., West Grove, PA. The antibody is radioiodinated using the standard chloramine-T method. The antibody will bind to the Fc portion of any TRAIL-R/Fc protein that has bound to the cells. In all assays, non-specific binding of $^{125}$I-antibody is assayed in the absence of TRAIL-R/Fc, as well as in the presence of TRAIL-R/Fc and a 200-fold molar excess of unlabeled mouse anti-human IgG antibody.

Cell-bound $^{125}$I-antibody is quantified on a Packard Autogamma counter. Affinity calculations (Scatchard, *Ann. N.Y. Acad. Sci.* 51:660, 1949) are generated on RS/1 (BBN Software, Boston, MA) run on a Microvax computer.

Another type of suitable binding assay is a competitive binding assay. To illustrate, biological activity of a TRAIL-R variant may be determined by assaying for the variant's ability to compete with a native TRAIL-R for binding to TRAIL.

Competitive binding assays can be performed by conventional methodology. Reagents that may be employed in competitive binding assays include radiolabeled TRAIL-R and intact cells expressing TRAIL (endogenous or recombinant) on the cell surface. For example, a radiolabeled soluble TRAIL-R fragment can be used to compete with a soluble TRAIL-R variant for binding to cell surface TRAIL. Instead of intact cells, one could substitute a soluble TRAIL/Fc fusion protein bound to a solid phase through the interaction of Protein A or Protein G (on the solid phase) with the Fc moiety. Chromatography columns that contain Protein A and Protein G include those available from Pharmacia Biotech, Inc., Piscataway, NJ. Another type of competitive binding assay utilizes radiolabeled soluble TRAIL, such as a soluble TRAIL/Fc fusion protein, and intact cells expressing TRAIL-R. Qualitative results can be obtained by competitive autoradiographic plate binding assays, while Scatchard plots (Scatchard, *Ann. N.Y. Acad. Sci.* 51:660, 1949) may be utilized to generate quantitative results.

Another type of assay for biological activity involves testing a TRAIL-R polypeptide for the ability to block TRAIL-mediated apoptosis of target cells, such as the human leukemic T-cell line known as Jurkat cells, for example. TRAIL-mediated apoptosis of the cell line designated Jurkat clone E6-1 (ATCC TIB 152) is demonstrated in assay procedures described in PCT application WO 97/01633, hereby incorporated by reference.

<u>Uses of TRAIL-R</u>

Uses of TRAIL-R include, but are not limited to, the following. Certain of these uses of TRAIL-R flow from its ability to bind TRAIL.

TRAIL-R finds use as a protein purification reagent. TRAIL-R polypeptides may be attached to a solid support material and used to purify TRAIL proteins by affinity chromatography. In particular embodiments, a TRAIL-R polypeptide (in any form described herein that is capable of binding TRAIL) is attached to a solid support by conventional procedures. As one example, chromatography columns containing functional groups that will react with functional groups on amino acid side chains of proteins are available (Pharmacia Biotech, Inc., Piscataway, NJ). In an alternative, a TRAIL-R/Fc protein is attached to Protein A- or Protein G-containing chromatography columns through interaction with the Fc moiety.

TRAIL-R proteins also find use in measuring the biological activity of TRAIL proteins in terms of their binding affinity for TRAIL-R. TRAIL-R proteins thus may be employed by those conducting "quality assurance" studies, e.g., to monitor shelf life and stability of TRAIL protein under different conditions. To illustrate, TRAIL-R may

be employed in a binding affinity study to measure the biological activity of a TRAIL protein that has been stored at different temperatures, or produced in different cell types. TRAIL-R also may be used to determine whether biological activity is retained after modification of a TRAIL protein (e.g., chemical modification, truncation, mutation, etc.). The binding affinity of the modified TRAIL protein for TRAIL-R is compared to that of an unmodified TRAIL protein to detect any adverse impact of the modifications on biological activity of TRAIL. The biological activity of a TRAIL protein thus can be ascertained before it is used in a research study, for example.

TRAIL-R also finds use in purifying or identifying cells that express TRAIL on the cell surface. TRAIL-R polypeptides are bound to a solid phase such as a column chromatography matrix or a similar suitable substrate. For example, magnetic microspheres can be coated with TRAIL-R and held in an incubation vessel through a magnetic field. Suspensions of cell mixtures containing TRAIL-expressing cells are contacted with the solid phase having TRAIL-R thereon. Cells expressing TRAIL on the cell surface bind to the fixed TRAIL-R, and unbound cells then are washed away.

Alternatively, TRAIL-R can be conjugated to a detectable moiety, then incubated with cells to be tested for TRAIL expression. After incubation, unbound labeled TRAIL-R is removed and the presence or absence of the detectable moiety on the cells is determined.

In a further alternative, mixtures of cells suspected of containing $TRAIL^+$ cells are incubated with biotinylated TRAIL-R. Incubation periods are typically at least one hour in duration to ensure sufficient binding. The resulting mixture then is passed through a column packed with avidin-coated beads, whereby the high affinity of biotin for avidin provides binding of the desired cells to the beads. Procedures for using avidin-coated beads are known (see Berenson, et al. *J. Cell. Biochem.*, 10D:239, 1986). Washing to remove unbound material, and the release of the bound cells, are performed using conventional methods.

TRAIL-R polypeptides also find use as carriers for delivering agents attached thereto to cells bearing TRAIL. Cells expressing TRAIL include those identified in Wiley et al. (*Immunity*, 3:673-682, 1995). TRAIL-R proteins thus can be used to deliver diagnostic or therapeutic agents to such cells (or to other cell types found to express TRAIL on the cell surface) in *in vitro* or *in vivo* procedures.

Detectable (diagnostic) and therapeutic agents that may be attached to a TRAIL-R polypeptide include, but are not limited to, toxins, other cytotoxic agents, drugs, radionuclides, chromophores, enzymes that catalyze a colorimetric or fluorometric reaction, and the like, with the particular agent being chosen according to the intended

application. Among the toxins are ricin, abrin, diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A, ribosomal inactivating proteins, mycotoxins such as trichothecenes, and derivatives and fragments (e.g., single chains) thereof. Radionuclides suitable for diagnostic use include, but are not limited to, $^{123}I$, $^{131}I$, $^{99m}Tc$, $^{111}In$, and $^{76}Br$. Examples of radionuclides suitable for therapeutic use are $^{131}I$, $^{211}At$, $^{77}Br$, $^{186}Re$, $^{188}Re$, $^{212}Pb$, $^{212}Bi$, $^{109}Pd$, $^{64}Cu$, and $^{67}Cu$.

Such agents may be attached to the TRAIL-R by any suitable conventional procedure. TRAIL-R, being a protein, comprises functional groups on amino acid side chains that can be reacted with functional groups on a desired agent to form covalent bonds, for example. Alternatively, the protein or agent may be derivatized to generate or attach a desired reactive functional group. The derivatization may involve attachment of one of the bifunctional coupling reagents available for attaching various molecules to proteins (Pierce Chemical Company, Rockford, Illinois). A number of techniques for radiolabeling proteins are known. Radionuclide metals may be attached to TRAIL-R by using a suitable bifunctional chelating agent, for example.

Conjugates comprising TRAIL-R and a suitable diagnostic or therapeutic agent (preferably covalently linked) are thus prepared. The conjugates are administered or otherwise employed in an amount appropriate for the particular application.

Another use of the protein of the present invention is as a research tool for studying the biological effects that result from inhibiting TRAIL/TRAIL-R interactions on different cell types. TRAIL-R polypeptides also may be employed in *in vitro* assays for detecting TRAIL or TRAIL-R or the interactions thereof.

TRAIL-R may be employed in inhibiting a biological activity of TRAIL, in *in vitro* or *in vivo* procedures. A purified TRAIL-R polypeptide may be used to inhibit binding of TRAIL to endogenous cell surface TRAIL-R. Biological effects that result from the binding of TRAIL to endogenous receptors thus are inhibited. Various forms of TRAIL-R may be employed, including, for example, the above-described TRAIL-R fragments, oligomers, derivatives, and variants that are capable of binding TRAIL. In one embodiment, a soluble TRAIL-R is employed to inhibit a biological activity of TRAIL, e.g., to inhibit TRAIL-mediated apoptosis of particular cells.

TRAIL-R may be administered to a mammal to treat a TRAIL-mediated disorder. Such TRAIL-mediated disorders include conditions caused (directly or indirectly) or exacerbated by TRAIL.

TRAIL-R may be useful for treating thrombotic microangiopathies. One such disorder is thrombotic thrombocytopenic purpura (TTP) (Kwaan, H.C., *Semin. Hematol.*, 24:71, 1987; Thompson et al., *Blood*, 80:1890, 1992). Increasing TTP-

associated mortality rates have been reported by the U.S. Centers for Disease Control (Torok et al., *Am. J. Hematol.* 50:84, 1995).

Plasma from patients afflicted with TTP (including HIV$^+$ and HIV$^-$ patients) induces apoptosis of human endothelial cells of dermal microvascular origin, but not large vessel origin (Laurence et al., *Blood*, 87:3245, April 15, 1996). Plasma of TTP patients thus is thought to contain one or more factors that directly or indirectly induce apoptosis. As described in PCT application WO 97/01633 (hereby incorporated by reference), TRAIL is present in the serum of TTP patients, and may play a role in inducing apoptosis of microvascular endothelial cells.

Another thrombotic microangiopathy is hemolytic-uremic syndrome (HUS) (Moake, J.L., *Lancet*, 343:393, 1994; Melnyk et al., (*Arch. Intern. Med.*, 155:2077, 1995; Thompson et al., *supra*). One embodiment of the invention is directed to use of TRAIL-R to treat the condition that is often referred to as "adult HUS" (even though it can strike children as well). A disorder known as childhood/diarrhea-associated HUS differs in etiology from adult HUS.

Other conditions characterized by clotting of small blood vessels may be treated using TRAIL-R. Such conditions include but are not limited to the following. Cardiac problems seen in about 5-10% of pediatric AIDS patients are believed to involve clotting of small blood vessels. Breakdown of the microvasculature in the heart has been reported in multiple sclerosis patients. As a further example, treatment of systemic lupus erythematosus (SLE) is contemplated.

In one embodiment, a patient's blood or plasma is contacted with TRAIL-R *ex vivo*. The TRAIL-R may be bound to a suitable chromatography matrix by conventional procedures. The patient's blood or plasma flows through a chromatography column containing TRAIL-R bound to the matrix, before being returned to the patient. The immobilized receptor binds TRAIL, thus removing TRAIL protein from the patient's blood.

Alternatively, TRAIL-R may be administered *in vivo* to a patient afflicted with a thrombotic microangiopathy. In one embodiment, a soluble form of TRAIL-R is administered to the patient.

The present invention thus provides a method for treating a thrombotic microangiopathy, involving use of an effective amount of TRAIL-R. A TRAIL-R polypeptide may be employed in *in vivo* or *ex vivo* procedures, to inhibit TRAIL-mediated damage to (e.g., apoptosis of) microvascular endothelial cells.

TRAIL-R may be employed in conjunction with other agents useful in treating a particular disorder. In an *in vitro* study reported by Laurence et al. (*Blood* 87:3245,

1996), some reduction of TTP plasma-mediated apoptosis of microvascular endothelial cells was achieved by using an anti-Fas blocking antibody, aurintricarboxylic acid, or normal plasma depleted of cryoprecipitate.

Thus, a patient may be treated with an agent that inhibits Fas-ligand-mediated apoptosis of endothelial cells, in combination with an agent that inhibits TRAIL-mediated apoptosis of endothelial cells. In one embodiment, TRAIL-R and an anti-FAS blocking antibody are both administered to a patient afflicted with a disorder characterized by thrombotic microangiopathy, such as TTP or HUS. Examples of blocking monoclonal antibodies directed against Fas antigen (CD95) are described in PCT application publication number WO 95/10540, hereby incorporated by reference.

TRAIL-R of the present invention may be used in developing treatments for any disorder mediated (directly or indirectly) by defective, or insufficient amounts of, TRAIL-R. TRAIL-R polypeptides may be administered to a mammal afflicted with such a disorder.

Compositions comprising an effective amount of a TRAIL-R polypeptide of the present invention, in combination with other components such as a physiologically acceptable diluent, carrier, or excipient, are provided herein. TRAIL-R can be formulated according to known methods used to prepare pharmaceutically useful compositions. TRAIL-R can be combined in admixture, either as the sole active material or with other known active materials suitable for a given indication, with pharmaceutically acceptable diluents (e.g., saline, Tris-HCl, acetate, and phosphate buffered solutions), preservatives (e.g., thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable formulations for pharmaceutical compositions include those described in *Remington's Pharmaceutical Sciences*, 16th ed. 1980, Mack Publishing Company, Easton, PA.

In addition, such compositions can contain TRAIL-R complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of TRAIL-R, and are thus chosen according to the intended application. TRAIL-R expressed on the surface of a cell may find use, as well.

Compositions of the present invention may contain a TRAIL-R polypeptide in any form described herein, such as native proteins, variants, derivatives, oligomers, and biologically active fragments. In particular embodiments, the composition

comprises a soluble TRAIL-R polypeptide or an oligomer comprising soluble TRAIL-R polypeptides.

TRAIL-R can be administered in any suitable manner, e.g., topically, parenterally, or by inhalation. The term "parenteral" includes injection, e.g., by subcutaneous, intravenous, or intramuscular routes, also including localized administration, e.g., at a site of disease or injury. Sustained release from implants is also contemplated. One skilled in the pertinent art will recognize that suitable dosages will vary, depending upon such factors as the nature of the disorder to be treated, the patient's body weight, age, and general condition, and the route of administration. Preliminary doses can be determined according to animal tests, and the scaling of dosages for human administration are performed according to art-accepted practices.

<u>Antibodies</u>

Antibodies that are immunoreactive with TRAIL-R polypeptides are provided herein. Such antibodies specifically bind TRAIL-R, in that the antibodies bind to TRAIL-R *via* the antigen-binding sites of the antibody (as opposed to non-specific binding).

The TRAIL-R protein prepared as described in example 1 may be employed as an immunogen in producing antibodies immunoreactive therewith. Alternatively, another form of TRAIL-R, such as a fragment or fusion protein, is employed as the immunogen.

Polyclonal and monoclonal antibodies may be prepared by conventional techniques. See, for example, *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Kennet et al. (eds.), Plenum Press, New York (1980); and *Antibodies: A Laboratory Manual*, Harlow and Land (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1988). Production of monoclonal antibodies directed against TRAIL-R is further illustrated in example 3.

Antigen-binding fragments of such antibodies, which may be produced by conventional techniques, are also encompassed by the present invention. Examples of such fragments include, but are not limited to, Fab and $F(ab')_2$ fragments. Antibody fragments and derivatives produced by genetic engineering techniques are also provided.

The monoclonal antibodies of the present invention include chimeric antibodies, e.g., humanized versions of murine monoclonal antibodies. Such humanized antibodies may be prepared by known techniques, and offer the advantage of reduced immunogenicity when the antibodies are administered to humans. In one embodiment,

a humanized monoclonal antibody comprises the variable region of a murine antibody (or just the antigen binding site thereof) and a constant region derived from a human antibody. Alternatively, a humanized antibody fragment may comprise the antigen binding site of a murine monoclonal antibody and a variable region fragment (lacking the antigen-binding site) derived from a human antibody. Procedures for the production of chimeric and further engineered monoclonal antibodies include those described in Riechmann et al. (*Nature 332*:323, 1988), Liu et al. (*PNAS 84*:3439, 1987), Larrick et al. (*Bio/Technology 7*:934, 1989), and Winter and Harris (*TIPS 14*:139, May, 1993).

Among the uses of the antibodies is use in assays to detect the presence of TRAIL-R polypeptides, either *in vitro* or *in vivo*. The antibodies also may be employed in purifying TRAIL-R proteins by immunoaffinity chromatography.

Those antibodies that additionally can block binding of TRAIL-R to TRAIL may be used to inhibit a biological activity that results from such binding. Such blocking antibodies may be identified using any suitable assay procedure, such as by testing antibodies for the ability to inhibit binding of TRAIL to cells expressing TRAIL-R. Examples of such cells are the Jurkat cells and PS1 cells described in example 2 below. Alternatively, blocking antibodies may be identified in assays for the ability to inhibit a biological effect that results from binding of TRAIL to target cells. Antibodies may be assayed for the ability to inhibit TRAIL-mediated lysis of Jurkat cells, for example.

Such an antibody may be employed in an *in vitro* procedure, or administered *in vivo* to inhibit a TRAIL-R-mediated biological activity. Disorders caused or exacerbated (directly or indirectly) by the interaction of TRAIL with cell surface TRAIL receptor thus may be treated. A therapeutic method involves *in vivo* administration of a blocking antibody to a mammal in an amount effective in inhibiting a TRAIL-mediated biological activity. Disorders caused or exacerbated by TRAIL, directly or indirectly, are thus treated. Monoclonal antibodies are generally preferred for use in such therapeutic methods. In one embodiment, an antigen-binding antibody fragment is employed.

A blocking antibody directed against TRAIL-R may be substituted for TRAIL-R in the above-described method of treating thrombotic microangiopathy, e.g., in treating TTP or HUS. The antibody is administered *in vivo*, to inhibit TRAIL-mediated damage to (e.g., apoptosis of) microvascular endothelial cells.

Compositions comprising an antibody that is directed against TRAIL-R, and a physiologically acceptable diluent, excipient, or carrier, are provided herein. Suitable

components of such compositions are as described above for compositions containing TRAIL-R proteins.

Also provided herein are conjugates comprising a detectable (e.g., diagnostic) or therapeutic agent, attached to an antibody directed against TRAIL-R. Examples of such agents are presented above. The conjugates find use in *in vitro* or *in vivo* procedures.

Nucleic Acids and Uses Thereof

The present invention provides TRAIL-R nucleic acids. Such nucleic acids include, but are not limited to, DNA encoding the peptides described in example 2. Such DNAs can be identified from knowledge of the genetic code. One embodiment of the invention is directed to fragments of TRAIL-R nucleotide sequences comprising at least about 17 contiguous nucleotides of a TRAIL-R DNA sequence. Nucleic acids provided herein include DNA and RNA complements of said fragments, along with both single-stranded and double-stranded forms of the TRAIL-R DNA.

Among the uses of TRAIL-R nucleic acid fragments is use as probes or primers. Using knowledge of the genetic code in combination with the amino acid sequences set forth in example 2, sets of degenerate oligonucleotides can be prepared. Such oligonucleotides find use as primers, e.g., in polymerase chain reactions (PCR), whereby TRAIL-R DNA fragments are isolated and amplified.

The following examples are provided to further illustrate particular embodiments of the invention, and are not to be construed as limiting the scope of the present invention.

## EXAMPLE 1: Purification of TRAIL-R Protein

A human TRAIL receptor (TRAIL-R) protein was prepared by the following procedure. Trail-R was isolated from the cell membranes of Jurkat cells, a human acute T leukemia cell line. Jurkat cells were chosen because a specific band can be affinity precipitated from surface-biotinylated Jurkat cells, using Flag®-TRAIL covalently coupled to affi-gel beads (Biorad Laboratories, Richmond, CA). The precipitated band has a molecular weight of about 52 kD. A minor specific band of about 42 kD also was present, possibly accounting for a proteolytic breakdown product or a less glycosylated form of TRAIL-R.

Approximately 50 billion Jurkat cells were harvested by centrifugation (80 *ml* of cell pellet), washed once with PBS, then shock frozen on liquid nitrogen. Plasma

membranes were isolated according to method number three described in Maeda et al., *Biochim. et Biophys. Acta*, 731:115, 1983; hereby incorporated by reference) with five modifications:

1. The following protease inhibitors were included in all solutions at the indicated concentrations: Aprotinin, 150 nM; EDTA, 5 mM; Leupeptin, 1 μM; pA-PMSF, 20 μM; Pefabloc, 500 μM; Pepstatin A, 1 μM; PMSF, 500 μM.
2. Dithiothreitol was not used.
3. DNAase was not used in the homogenization solution.
4. 1.25 ml of homogenization buffer was used per ml of cell pellet.
5. The homogenization was accomplished by five passages through a ground glass dounce homogenizer.

After isolation of the cell membranes, proteins were solubilized by resuspending the isolated membranes in 50 ml PBS containing 1% octylglucoside and all of the above mentioned protease inhibitors at the above indicated concentrations. The resulting solution was then repeatedly vortexed during a thirty-minute incubation at 4°C. The solution was then centrifuged at 20,000 rpm in an SW28 rotor in an LE-80 Beckman ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) at 4°C for 30 minutes to obtain the supernatant (the membrane extract).

Chromatography

The first step of purification of TRAIL-R out of the membrane extract prepared above was affinity chromatography. The membrane extract was first applied to an anti-Flag® M2 affi-gel column (10 mg of monoclonal antibody M2 coupled to 2 ml of Affi-gel beads) to adsorb any nonspecifically binding material. The flow-through was then applied to a Flag®-TRAIL affi-gel column (10 mg of recombinant protein coupled to 1 ml of affi-gel beads).

The Affi-gel support is an N-hydroxysuccinimide ester of a derivatized, crosslinked agarose gel bead (available from Biorad Laboratories, Richmond, CA). As discussed above, the Flag® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, provides an epitope reversibly bound by specific monoclonal antibodies, enabling rapid assay and facile purification of expressed recombinant protein. M2 is a monoclonal antibody that binds Flag®. Monoclonal antibodies that bind the Flag® peptide, as well as other reagents for preparing and using Flag® fusion proteins, are available from Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Connecticut. Preparation of Flag®-TRAIL fusion proteins (comprising Flag® fused to a soluble

TRAIL polypeptide) is further described in PCT application WO 97/01633, hereby incorporated by reference.

The column was washed with 25 ml of each of the following buffers, in the order indicated:

1. PBS containing 1% octylglucoside
2. PBS
3. PBS containing an additional 200 mM NaCl
4. PBS

The bound material was eluted with 50 mM Na Citrate (pH 3) in 1 ml fractions and immediately neutralized with 300 µl of 1 M Tris-HCl (pH 8.5) per fraction. The TRAIL-binding activity of each fraction was determined by a TRAIL-R-specific ELISA as described below. Fractions with high TRAIL-binding activity were pooled, brought to 0.1 % Trifluoroacetic acid (TFA), and subsequently chromatographed on a capillary reversed-phase HPLC column [500 µm internal diameter X 25 cm fused silicone capillary column packed with 5 µm Vydac $C_4$ material (Vydac, Hesperia, CA)] using a linear gradient (2% per minute) from 0% to 100% acetonitrile in water containing 0.1% TFA. Fractions containing high TRAIL-binding activity are then determined as above, pooled, and, if desired, lyophylized.

TRAIL-R-specific ELISA:

Serial dilutions of TRAIL-R-containing samples (in 50 mM $NaHCO_3$, brought to pH 9 with NaOH) were coated onto Linbro/Titertek 96 well flat bottom E.I.A. microtitration plates (ICN Biomedicals Inc., Aurora, OH) at 100 µl/well. After incubation at 4°C for 16 hours, the wells were washed six times with 200 µl PBS containing 0.05% Tween-20 (PBS-Tween). The wells were then incubated with Flag®-TRAIL at 1 µg/ml in PBS-Tween with 5% fetal calf serum (FCS) for 90 minutes (100 µl per well), followed by washing as above. Next, each well was incubated with the anti-Flag® monoclonal antibody M2 at 1 µg/ml in PBS-Tween containing 5% FCS for 90 minutes (100 µl per well), followed by washing as above. Subsequently, wells were incubated with a polyclonal goat anti-mIgG1-specific horseradish peroxidase-conjugated antibody (a 1:5000 dilution of the commercial stock in PBS-Tween containing 5% FCS) for 90 minutes (100 µl per well). The HRP-conjugated antibody was obtained from Southern Biotechnology Associates, Inc., Birmingham, Alabama. Wells then were washed six times, as above.

For development of the ELISA, a substrate mix [100 µl per well of a 1:1 premix of the TMB Peroxidase Substrate and Peroxidase Solution B (Kirkegaard Perry

Laboratories, Gaithersburg, Maryland)] was added to the wells. After sufficient color reaction, the enzymatic reaction was terminated by addition of 2 N $H_2SO_4$ (50 µl per well). Color intensity (indicating TRAIL-binding activity) was determined by measuring extinction at 450 nm on a V Max plate reader (Molecular Devices, Sunnyvale, CA).

## EXAMPLE 2: Amino Acid Sequence

(a) TRAIL-R purified from Jurkat cells

TRAIL-R protein isolated from Jurkat cells was digested with trypsin, using conventional procedures. Amino acid sequence analysis was conducted on one of the peptide fragments produced by the tryptic digest. The fragment was found to contain the sequence:

VPANEGD

(b) TRAIL-R purified from PS-1 cells

TRAIL-R protein was also isolated from PS-1 cells. PS-1 is a human B cell line that spontaneously arose after lethal irradiation of human peripheral blood lymphocytes (PBLs). The TRAIL-R protein was digested with trypsin, using conventional procedures. Amino acid sequence analysis was conducted on peptide fragments that resulted from the tryptic digest. One of the fragments was found to contain the sequence:

VPANEGD

Two other fragments were found to contain the following amino acid sequences, respectively:

VCEC
SGEVELSSV

## EXAMPLE 3: Monoclonal Antibodies That Bind TRAIL-R

This example illustrates a method for preparing monoclonal antibodies that bind TRAIL-R. Suitable immunogens that may be employed in generating such antibodies include, but are not limited to, purified TRAIL-R protein or an immunogenic fragment

thereof such as the extracellular domain, or fusion proteins containing TRAIL-R (e.g., a soluble TRAIL-R/Fc fusion protein).

Purified TRAIL-R can be used to generate monoclonal antibodies immunoreactive therewith, using conventional techniques such as those described in U.S. Patent 4,411,993. Briefly, mice are immunized with TRAIL-R immunogen emulsified in complete Freund's adjuvant, and injected in amounts ranging from 10-100 μg subcutaneously or intraperitoneally. Ten to twelve days later, the immunized animals are boosted with additional TRAIL-R emulsified in incomplete Freund's adjuvant. Mice are periodically boosted thereafter on a weekly to bi-weekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision to test for TRAIL-R antibodies by dot blot assay, ELISA (Enzyme-Linked Immunosorbent Assay) or inhibition of TRAIL binding.

Following detection of an appropriate antibody titer, positive animals are provided one last intravenous injection of TRAIL-R in saline. Three to four days later, the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line, e.g., NS1 or preferably P3x63Ag8.653 (ATCC CRL 1580). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells are screened by ELISA for reactivity against purified TRAIL-R by adaptations of the techniques disclosed in Engvall et al., *Immunochem.* 8:871, 1971 and in U.S. Patent 4,703,004. A preferred screening technique is the antibody capture technique described in Beckmann et al., (*J. Immunol.* 144:4212, 1990) Positive hybridoma cells can be injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-TRAIL-R monoclonal antibodies. Alternatively, hybridoma cells can be grown *in vitro* in flasks or roller bottles by various techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to Protein A or Protein G can also be used, as can affinity chromatography based upon binding to TRAIL-R.

What is claimed is:

1. A purified TRAIL receptor (TRAIL-R) polypeptide that is capable of binding TRAIL, wherein the TRAIL-R is characterized as comprising the amino acid sequence VPANEGD.

2. A TRAIL-R polypeptide of claim 1, wherein said polypeptide is further characterized by a molecular weight of about 50 to 55 kilodaltons.

3. A purified TRAIL-R polypeptide that is capable of binding TRAIL, wherein the TRAIL-R is characterized as comprising at least one of the amino acid sequences selected from the group consisting of VPANEGD, VCEC and SGEVELSSV.

4. A TRAIL-R polypeptide of claim 3, wherein said polypeptide is further characterized by a molecular weight of about 50 to 55 kilodaltons.

5. A purified TRAIL-R polypeptide, wherein said polypeptide is a fragment of the TRAIL-R polypeptide of claim 1.

6. A purified TRAIL-R polypeptide, wherein said polypeptide is a fragment of the TRAIL-R polypeptide of claim 3.

7. A TRAIL-R polypeptide of claim 5, wherein said fragment is a soluble TRAIL-R polypeptide.

8. A TRAIL-R polypeptide of claim 6, wherein said fragment is a soluble TRAIL-R polypeptide.

9. An oligomer comprising from two to four TRAIL-R polypeptides of claim 1, 3, 7, or 8.

10. An oligomer of claim 9, wherein said oligomer is a dimer comprising two soluble TRAIL-R/Fc fusion proteins.

11. A composition comprising a TRAIL-R polypeptide of claim 1, 2, 3, 4, 5, 6, 7, or 8, and a physiologically acceptable diluent, excipient, or carrier.

12. A composition comprising an oligomer of claim 9 and a physiologically acceptable diluent, excipient, or carrier.

13. An antibody that is directed against a TRAIL-R polypeptide of claim 1 or 3.

14. An antibody of claim 13, wherein the antibody is a monoclonal antibody.